MANNOSE ACTIVATION PATHWAY FOR GLYCOCONJUGATE BIOSYNTHESIS IS INDISPENSABLE FOR *LEISHMANIA* VIRULENCE – A STUDY THAT IDENTIFIES POTENTIAL DRUG TARGETS AND VACCINE DEVELOPMENT CANDIDATES

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I. **Attila Garami** and Thomas Ilg: The role of phosphomannose isomerase in *Leishmania mexicana* glycoconjugate synthesis and virulence. ***J. Biol. Chem.*** 2001 Mar 2; 276(9): 6566-75.

II. **Attila Garami** and Thomas Ilg: Disruption of mannose activation in *Leishmania mexicana*: GDP-mannose pyrophosphorylase is required for virulence, but not for viability. ***EMBO J.*** 2001 Jul 16; 20(14): 3657-66.

III. **Attila Garami,** Angela Mehlert and Thomas Ilg: Glycosylation defects and virulence phenotypes of *Leishmania mexicana* phosphomannomutase and dolicholphosphate-mannose synthase gene deletion mutants. ***Mol. Cell. Biol.*** 2001 Dec; 21(23): 8168-83.

CONTENTS

1. Summary

Leishmaniasis is a vector-borne parasitic disease of tropical and subtropical regions caused by the obligate intracellular protozoan parasite *Leishmania* and currently threatens over 350 million lives in 88 countries around the world with an estimated prevalence of 12 million and incidence of 1.5-2 million. It also affects inhabitants of European subtropical regions, where a more fatal form of the disease occurs caused by co-infections of *Leishmania*/HIV. Since control strategies for the treatment and eradication of leishmaniasis improved little over the past decades, research driven advancements in diagnosis, treatment and prevention are prime necessities.

The remarkable survival capabilities of *Leishmania*, the successful establishment of infection and multiplication in hostile environments of both the digestive tract of the sandfly vector and macrophages of mammalian host, are largely attributed to macromolecules that form a protective shield, the glycocalyx, around the parasite. This coat consists of surface-bound or optionally secreted mannose-rich glycoconjugates that are thought to be key determinants of *Leishmania* viability and virulence.

The building blocks for the assembly of different glycoconjugates are the activated nucleotide sugar donors, such as GDP-mannose and Dol-P-mannose. The activation of mannose from Man-6-PO_4 to GDP-mannose or Dol-P-mannose donors requires the sequential enzymatic action of phosphomannomutase (PMM), GDP-mannose pyrophosphorylase (GDPMP), and Dol-P-mannose synthase (DPMS). Optionally the hexose-monophosphate pool derived Frc-6-PO_4 can also be converted to Man-6-PO_4 in a reaction catalyzed by phosphomannose isomerase (PMI), to feed the mannose activation pathway.

Given the high rate of glycoconjugate synthesis in *Leishmania*, the proper mannose supply is predicted to be of prime importance for these parasites. Therefore, in our studies we tried to elucidate the importance of the mannose activation pathway by a reverse genetics approach, and to estimate its specific impact on *Leishmania* viability, growth, glycoconjugate synthesis, and virulence that eventually may lead to identification of new drug targets and novel treatments for leishmaniasis.

The genes of *Leishmania mexicana* mannose activation pathway were homology cloned and sequenced to generate *Leishmania* mutants lacking function of the enzyme cascade at different levels. Remarkably, in contrast to different pathogenic fungi, yeast species or humans – wherein functional lack of mannose activation pathway, leading to complete lack of glycoconjugates, is incompatible with life – in *Leishmania*, targeted gene deletion still led to viable mutants. These results doubtlessly showed that mannose-containing glycoconjugates, in contrast to a former report (Ilgoutz, 1999, EMBO J) also in *L. mexicana*, are not essential for *Leishmania* viability and

therefore may provide a unique model system for studies on eukaryotic glycoconjugate assembly. As it was anticipated, the mannose activation pathway mutants showed a complex phenotype with the lack of different mannose-containing glycoconjugates, which affected *Leishmania* virulence to various extents. The mutants lacking PMI or DPMS enzymes remained infectious, although were attenuated, while PMM or GDPMP lacking mutants were found to be avirulent.

Our results identify the mannose activation pathway as a virulence pathway in *Leishmania* that consequently may serve as an appealing potential drug target for combating leishmaniasis. In contrast to earlier unsuccessful attempts to unfold the mystery of *Leishmania* virulence by targeting single mannose-containing glycoconjugates, a new view is arising based on our novel approach, where the pathway of glycoconjugate biosynthesis was targeted. This novel view strongly suggests that instead of a single mannose-containing glycoconjugate, the various redundant glycan motifs, which are shared between different classes of glycoconjugates may be responsible for the evolutionarily successful virulence of *Leishmania*.

The avirulent *Leishmania* strains described in this study may also be potentially further developed to efficient vaccines for the prevention of leishmaniasis.

2. Összefoglalás

Leishmaniasis a trópusi és szubtrópusi területek endémiás, vektor terjeszetette parazitás betegsége, amelyet az obligát intracelluláris protozoon parazita *Leishmania* okoz és amely jelenleg többmint 350 millió ember életét veszélyezteti a világ mintegy 88 országában, körülbelül 12 milliós becsült prevalenciával és 1,5-2 milliós incidenciával. A betegség szintén megtalálható Európa szubtrópusi területein, ahol egy sokkal végzetesebb kimenetelû formája fordul elô, amelyet *Leishmania*/HIV együttes fertôzése okoz. Mivel az elmúlt évtizedek alatt a leishmaniasis kezelésének és eradikációjának a kontrol stratégiái kevéssé fejlôdek, a kutatás ösztönözte elôrehaladás a diagnózis és prevenció területén elsôdleges fontosságú.

A *Leishmania* hihetetlen túlélôképessége, sikeres fertôzô képessége és szaporodása, mind a lepkeszúnyog vektor emésztôrendszerének és az emlôs gazda makrofágjainak kedvezôtlen körülményei között, nagyrészt a parazita felszínén található makromolekulák által formált védôpajzsnak, a glikokálixnak, tulajdonítható. Ez a réteg, felszínhez kötött vagy esetlegesen szekretált mannózban gazdag glikokonjugátokból áll, amely molekulákat a *Leishmania* életképességének és virulenciájának kulcsfontosságú meghatározójaként tartanak számon.

A különféle glikokonjugátok összeszereléséhez az építôkövek az aktivált nukleotidcukor donorok, mint a GDP-mannóz és dolikol-P-mannóz. A mannóz mannóz-6-PO_4-ból GDP-mannózzá vagy dolikol-P-mannózzá való aktiváláshoz enzimek sorának – foszfomannomutáz, GDP-mannóz-

pirofoszforiláz és dolichol-foszfomannóz mutáz – összehangolt mûködésére van szükség. Esetlegesen a hexóz-monofoszfát raktárból származó Frc-6-PO_4 is táplálhatja a mannóz aktiválás biokémiai útját, amely szintén átalakulhat Man-6-PO_4-tá egy a foszfomannóz-izomeráz katalizálta reakció révén.

A *Leishmania* glikokonjugátok nagysebességû szintézise miatt a megfelelô mannóz ellátás valószínûleg elsôdleges fontosságú a paraziták számára. Ezért kísérleteinkben próbáltuk tisztázni a mannóz aktiválás biokémiai útjának jelentôségét reverz genetikai megközelítés segítségével, és így felbecsülni annak jelentôségét a *Leishmania* életképessége, növekedése, glikokonjugát szintézise és virulenciája szempontjából. – ami végül új gyógyszercélpontok azonosításához és a leishmaniasis újszerû kezeléséhez is vezethet.

Leishmania mexicana mannóz aktiválás biokémiai útjának génjeit homológiájuk alapján klónoztuk, majd szekvenáltuk, azért hogy lépésenként ennek az enzimláncolatnak a különbözô szintjein levô hiánnyal rendelkezô mutánsokat tudjunk elôállítani. Rendkívûli módon, ellenben különféle kórokozó gomba és élesztôgomba fajokkal valamint emberrel – amelyekben a mannóz aktiválás biokémiai útjának hiánya, amely a glikokonjugátok teljes hiányához vezet, az élettel összeegyeztethetetlen – *Leishmaniá*ban génjeinek specifikus hiánya mégis életképes mutánsokat eredményez. Ezek az eredmények kétségtelenül bizonyítják, hogy a mannóz-tartalmú glikokonjugátok, ellenben egy korábbi beszámolóval (Ilgoutz, 1999, EMBO J) szintén *L. mexicana*ban, nem alapvetôen fontosak a *Leishmania* életképességéhez, ezért azok kitûnô modellrendszerként szolgálhatnak az eukarióta glikokonjugát szintézis tanulmányozására. Amint reméltük, a mannóz aktiválás biokémiai útjának mutánsai egy összetett fenotípust mutattak, a különféle mannóz-tartalmú glikokonjugátok hiányával, amely különbözô mértékben befolyásolta a *Leishmania* virulenciáját. Mutánsok amelyekbôl a foszfomannóz-izomeráz vagy a dolichol-foszfomannóz-mutáz hiányzott fertôzôképesek maradtak, bár legyöngítettek, míg a foszfomannomutáz vagy a GDP-mannóz-pirofoszforiláz hiányos mutánsok fertõzésképtelennek bizonyultak.

Eredményeink a mannóz aktiválás biokémiai útját, mint virulencia biokémiai útat azonosítják *Leishmaniá*ban, ami következésképpen potenciálisan vonzó gyógyszer-célpontként szolgálhat a leishmaniasis elleni küzdelemben. Ellenben a korábbi sikertelen próbálkozásokkal a *Leishmania* virulanciája titkának feltárására – amikoris egyes mannóz-tartalmú glikokonjugátokat céloztak – egy új nézet körvonalazódik újszerû megközelítésünk alapján, amely a glikokonjugátok bioszintézisének útját célozta. Ez az új nézet erôsen sugallja, hogy az egyes mannóz-tartalmú glikokonjugátok helyett, azok különbözô nagyszámban jelenlévô glikán motívumai – amelyek

részben közösek a glikokonjugátok különféle osztályaiban – lehetnek felelôsek a *Leishmania* evolúciósan sikeres virulenciájáért.

Az avirulens *Leishmania* mutáns törzsek, amelyek ebben a tanulmányban leírásra kerültek, potenciálisan továbbfejleszthetôek lehetnek hatékony oltóanyaggá a leishmaniasis megelôzésére.

3. Introduction

3.1 A broader context of tropical diseases

Since 'The Great White of Lambaréné' established his hospital in a province of French Equatorial Africa, nowadays Gabon, the global awareness for public health problems of the Third World still has not been sufficiently improved. Via his testimony, Albert Schweitzer called the attention of Nations on the must of easing the Third World people's misery. Ultimately, his devoted life was recognized by Nobel Peace Prize in 1952 for all the efforts on behalf of the 'Brotherhood of Nations'.

Over decades, hundreds and thousands had been devoted their lives to relieve the pain of the poorest; despite of these efforts the public health situation of the Third World is still unresolved. In fact, recently WHO has reported a further increase in the burden of tropical diseases. These facts define tropical research and innovative translation of its breakthrough achievements as essential activities in combating diseases of the Third World. Unfortunately, prioritization of research funding is still more than unsatisfactory, since only about 10% of global research funds are allocated to neglected tropical diseases that make up approximately 90% of the disease burden in our planet (Global Forum for Health Research, 2002).

Besides the dedicated efforts of WHO, the pharmaceutical industry still seems to be divided in giving support for easing such a burning health issues of the poorest. However, a positive trend is being outlined recently, showing that some pharmaceutical key players are becoming committed to support the Third World's health. While sacrificing bits of their profit, they can decently ease the burden of tropical diseases via supplying medicines at production cost or funding non-profit research institutes for combating tropical diseases. At the same time, WHO needs to convince others via long-lasting lobbying about just simply further producing some of the first line drugs of the medical arsenal against tropical diseases.

If there are still some doubts about the necessity of dealing with these emerging diseases, it should not be forgotten, that just a century ago Europe also knew these diseases very well. Moreover, according to recent scenarios of global climate change, we may easily face them again in the near future, since increasing temperature and rainfall is shown to be associated with increased

number of epidemics for several tropical diseases (Global environmental change, WHO website). Additionally, from an epidemiological aspect, the high risk of rapid diseases spread in our "global village" spiced with decent worldwide migration trends, also needs the attention of professionals.

Some serious problems are already at the doorstep, such as the world widely increasing incidence of drug resistant bugs, the frequency of opportunistic HIV co-infections and the lack of preventive vaccination. These global challenges certainly call for global solutions.

Taken together, tropical research that elucidates new cellular processes and identifies novel drug targets that can lead to more efficient treatment strategies and better diagnostics is of prime importance for the control of tropical diseases, especially for the citizens of the Third World. Of course, the ultimate global answer for the tropical disease burden, in alignment with WHO efforts, is the development of efficient vaccines for the prevention of these maladies.

3.2 Leishmaniasis a parasitic protozoan disease of tropics and subtropics

Three tropical diseases - African trypanosomiasis, Dengue fever and Leishmaniasis –belong to the highest priority category according to the disease burden, epidemiology trend and health economy based prioritization list of WHO (Remme JH, 2002).

Leishmaniasis is a vector-borne parasitic disease of tropical and subtropical regions of both the Old and New World. The causative agents of leishmaniasis are obligate intracellular protozoan species belonging to the *Leishmania* genus that are transmitted by the bite of a 2-3 millimeter-long insect vector, the sandfly (*Phlebotomus* or *Lutzomyia*, in the Old World or New World respectively) during blood feeding. *Leishmania* currently threaten over 350 million lives in 88 countries around the world with an estimated prevalence of 12 million, incidence of 1.5-2 million and disability adjusted life years (DALY) lost of 2.4 million (Fig.1). Leishmaniasis also affects citizens from subtropical regions of European countries such as France, Italy, Greece, Malta, Spain and Portugal. Indeed the incidence of leishmaniasis is sharply increasing in these regions that mainly consist of a more fatal form of the disease caused by co-infections of *Leishmania*/HIV (Fig.1). *Leishmania* infection can lead to diverse animal and human diseases, which clinically exhibit a rather wide spectrum from cutaneous, the mostly skin localized to visceral the generalized forms. The clinical pathology of *Leishmania* infection is determined by several factors of the "infectious triangle", namely by the host immune system and nutritional status, the given *Leishmania* species involved and the characteristics of the sandfly vector.

The most common form of the disease is cutaneous leishmaniasis, also known as espundia, which develops single skin ulcers on the most exposed, easily sandfly-bitten parts of the body, such as the ears, face, neck and extremities. This lesion usually remains localized and typically heals

spontaneously within 6 months to 2 years, but can also be accompanied by enormous tissue destruction and scarring eventually leading to stigmatization and social prejudice of the patient.

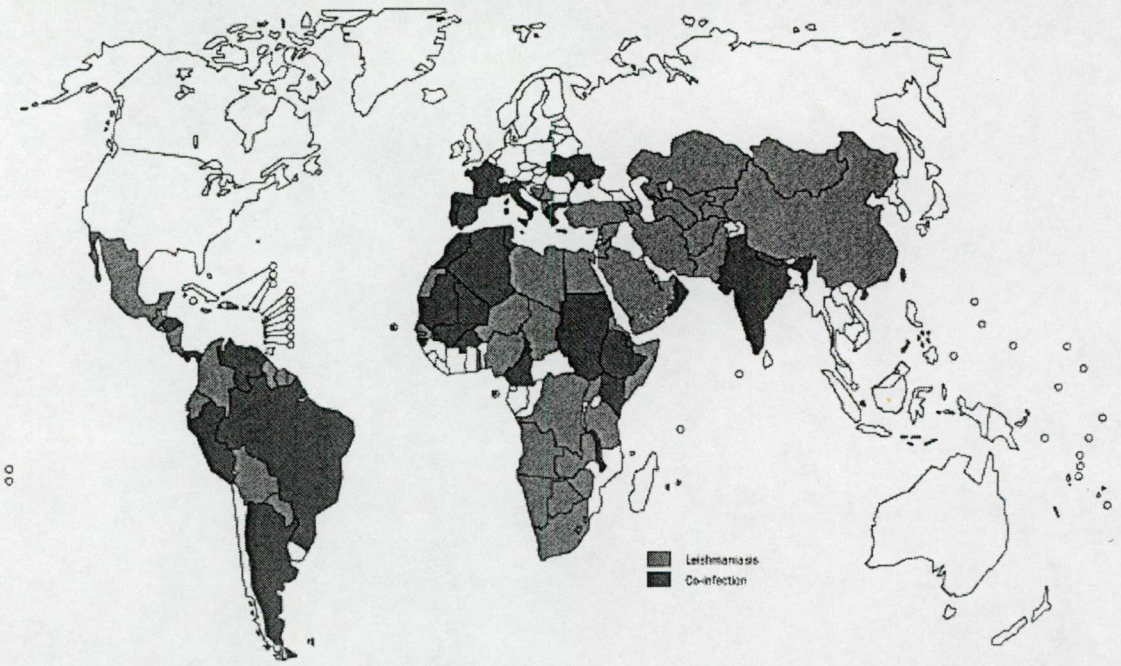


Fig.1. Global distribution of reported cases of leishmaniasis and *Leishmania*/HIV co-infection (source: Leishmaniasis and HIV co-infection, WHO Homepage)

Cutaneous leishmaniasis sometime is not limited to a single location, but develops numerous small, disseminated nodular skin lesions without self-healing potential. Developing this form of leishmaniasis, the diffuse cutaneous Leishmaniasis (DCL), is highly dependent on host factors such as the immune status or inherited genetic susceptibility loci, but to a limited extent, it is also influenced by the *Leishmania* species involved in the infection. Cutaneous leishmaniasis is predominantly caused by *L. major/L. tropica* and *L. mexicana/L. amazonensis* species in the Old and New Worlds respectively.

The mucocutaneous form of Leishmaniasis is caused by several South-American *Leishmania* species, most frequently by *L. brasiliensis*. Lesions predominantly occur on mucous membranes of the nose, mouth, throat cavities and the surrounding tissues. These lesions often lead to partial or total destruction of soft tissues that resembles those of lepromatous leprosy and result in severe scarring, disfiguring and frequently consequent humiliation and casting out from the society.

The visceral form of leishmaniasis, also known as kala azar, is the most devastating, which unless treated, leads to lethality approaching 100% (Molyneux DH, 1983; Leishmaniasis, WHO website). In this form, the primary sites of active infection are the spleen, liver and bone marrow, but parasites widely spread in the whole body via the infected immune cells and body fluids. Visceral leishmaniasis is clinically characterized by high fever, severe weight loss, hepato-

splenomegaly, and occasionally severe anemia that non-specific symptoms make diagnosis rather difficult. Nowadays, the most emerging type of visceral leishmaniasis occurs in HIV-infected individuals as severe opportunistic infection (Wolday D, 1999). Most of these *Leishmania*/HIV co-infected patients develop a considerably serious, fulminant and rapidly progressing form of visceral leishmaniasis, caused by a synergistic attack against the immune system. The causative agents of visceral form are predominantly two species, the *L. donovani* and *L. infantum/L. chagasi.*

Human cases of leishmaniasis are usually zoonoses, when the parasite is transmitted from reservoir species such as dogs, rodents, foxes, jackals and wild rodents (gerbils, sloths and opossums) to humans via the bite of the sandfly vector when feeding a blood meal. However, anthroponotic, from human to human, transmission of infection also occurs which is mediated by sandfly vectors. Examples for such an anthroponotic transmission are the urban cutaneous leishmaniasis caused by *L. tropica* and the visceral leishmaniasis in India caused by *L. donovani*, where sandfly mediated human-to-human transmission is indeed the major source of disease spread.

3.3 Leishmaniasis control strategies

Control strategies for the treatment and eradication of leishmaniasis has improved little over the past decades. Although some recently launched WHO programs facilitated advancements in diagnosis, treatment and prevention of leishmaniasis, proper disease control is still far from being satisfactory. Furthermore, no defined, safe vaccines yet exist for the prevention of leishmaniasis and only few drugs are available for treatment (Croft SL, 1997; Davies CR, 2003). For about half a century, pentavalent antimonials have been used as first line drugs for the treatment of all forms of leishmaniasis. Parasite resistance to these drugs has increased over decades and reached a significant level by now. Therefore, treatment with second line drugs, such as amphotericin B and pentamidine is more frequent, but unfortunately, they carry notable risk of serious toxic side effects. Remarkably, a "reinvented" application of an originally used anticancer drug, miltefosine, is in the late phase of clinical development or registered already in some countries (e.g. India) and shows high success rates of healing in both, visceral and cutaneous leishmaniasis. However, care should be taken since miltefosine is teratogenic in animal models, therefore it should not be prescribed to pregnant woman or to those of childbearing age without effective birth control. An additional shadow that falls on this success story is the appearance of miltefosine resistant *Leishmania* strains, which indeed underlines the need of testing multiple drug combination regiments to prevent emergence of parasite resistance to novel medicines.

3.4 Biology of *Leishmania*

Leishmania species evolutionarily represent one of the earliest eukaryotic organisms that possess mitochondria (Leipe DD, 1993) and form the genus *Leishmania* that taxonomically belongs to Trypanosomatidae family, Trypanosomatina suborder, and Kinetoplastida order (Fig.2). These ancient unicellular eukaryotic organisms have several peculiar cellular organelles (e.g. kinetoplast – that also refers to the order, and glycosomes) with unique cellular functions. Numerous morphologically distinct developmental forms of a given *Leishmania* species exist, which reflects adaptation to diverse environments of their digenetic life cycle. Principally, *Leishmania* alternate between the colonization of an insect vector by the extracellular, slender shape, flagellated and motile promastigotes, and that of a mammalian host by the obligatory intracellular, non-flagellated, round shape amastigotes (Fig.3).

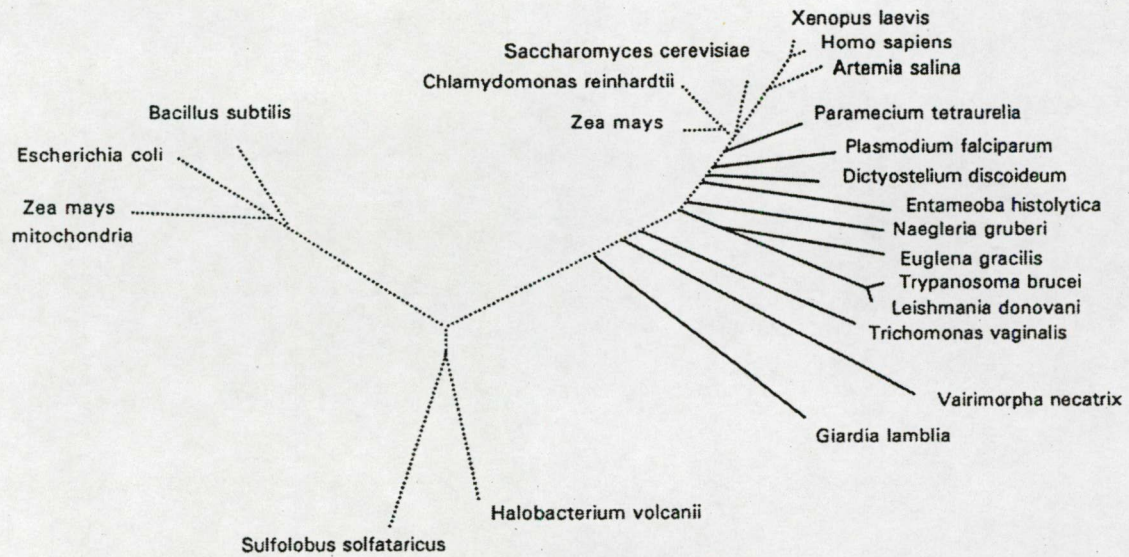


Fig.2. Phylogenetic tree based on the analysis of 16S rRNA sequences showing the evolutionary distance between organisms (Sogin ML, 1989).

Female sandflies acquire the parasites during blood feeding when they bite a *Leishmania* infected host. Ingested *Leishmania* reside within a peritrophic membrane that covers the blood meal in the sandfly intestine, here they transform to procyclic promastigotes, the short, motile flagellated form. Shortly after the peritrophic membrane is disrupted, the parasites acquire a slightly longer, slender shape and transform to the nectomonad promastigote form. The nectomonads are released to the lumen of the midgut, where with their flagella they intercalate between the microvilli of the epithelial cells and start to replicate in the mid-gut lumen against the harsh environment surrounding them. From this stage, via the free-swimming or cuticule attached haptomonad promastigote form they transform to highly motile, free-swimming, non-dividing metacyclic promastigotes that are highly infective and migrate to the sandfly foregut, where they get prepared for transmission. Near

the mouthpart, they start to secrete highly glycosylated macromolecules that form a "plug", which causes regurgitation, a functional disturbance of the sandfly digestive tract (Rogers ME, 2004). This strategy of the highly infective metacyclic promastigotes significantly increases the success of transmission to a new host.

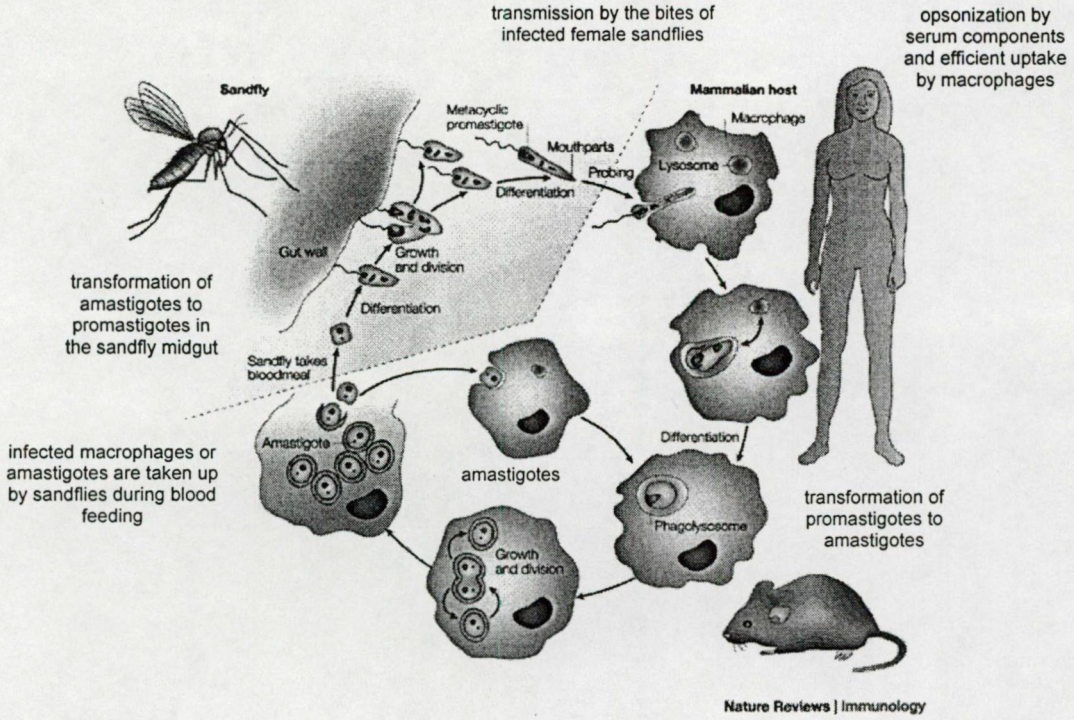


Fig.3. *Leishmania* life cycle (adapted from Sacks D, 2002).

In the skin of the vertebrate host, the freshly inoculated *Leishmania* get rapidly phagocytozed by macrophages or dendritic cells. These infected cells, when they migrate, carry *Leishmania* around the body and so facilitate the further spread of parasites via a 'by-stander' effect. Inside the host cell, triggered by the harshly changing environment, promastigotes rapidly (within 12-24 hours) transform to amastigotes. This short, non-motile, intracellular form of *Leishmania* is able to block the normal maturation of phagolysosomes and rapidly multiply under the surrounding hostile conditions that progressively result in the formation of parasitophorous vacuoles. This process leads to the gradual expansion of parasitophorous vacuoles that eventually rupture and the highly infective amastigotes are released to the body fluids and can colonize new host cells.

The remarkable survival capabilities of *Leishmania* throughout its life cycle, such as successful establishment of infection via subverting the innate and adaptive immune system and multiplication in hostile environments of both the sandfly digestive tract and host macrophages, are largely attributed to molecules that predominantly form a developmentally distinct surface coat, the glycocalyx, as a protective shield around the parasite. These primarily surface-bound or optionally

secreted sugar-rich glycoconjugate molecules are thought to be key determinants of *Leishmania* virulence via multiple functions attributed to them.

3.5 Immunology of *Leishmania* infection

To ensure its survival and reproduction in the host organism, *Leishmania* must compromise the defense mechanisms of the host immune system. This includes some rather unspecific ways for targeting the innate immune system and more sophisticated and less understood approaches for the derangement of the delicate balance of cellular Th1 and Th2 responses of the adaptive immune system (Reiner SL, 1995). In all these processes, surface or secreted glycoconjugate molecules are assumed to be involved, as key players.

3.5.1 Subversion of innate immune system by *Leishmania*

On each bite, during blood feeding of *Leishmania*-infected sandflies, approximately 10-100 metacyclic, highly infective promastigotes are inoculated into the skin of the mammalian host, where the parasites immediately encounter the humoral defense mechanisms of the innate immune system, specifically the alternative and lectin pathways of the complement system. Both metacyclic promastigotes and amastigotes are resistant to complement lysis that is believed to be conferred by different surface glycoconjugates (Mosser DM, 1985; Sacks DL, 1989).

In the host, metacyclic promastigotes immediately activate the alternative complement pathway, which leads to binding of C3 convertase activated C3 (C3b) to an abundant glycoconjugate component of the *Leishmania* glycocalyx coat, the lipophosphoglycan (LPG). Although this leads to the subsequent recruitment of the membrane attack complex (MAC), the lethal process of MAC insertion to the plasma membrane appears to be hampered by the specifically thick layer of LPG that can physically inhibit the direct access of MAC to the parasite surface (Hall BF, 1991). Shielding of promastigotes from the humoral defense is further supported by a surface metalloproteinase, the leishmanolysin (gp63) an enzyme that can rapidly convert the active LPG-bound C3b into an inactive form, iC3b (Brittingham A, 1996; Joshi PB, 1998), thus undermining the further activation of the complement cascade. On the other hand, C3b and iC3b also facilitate the attachment of opsonised parasites to macrophages and skin dendritic cell complement receptors CR1 and CR3 respectively (reviewed in Hall BF, 1991; Brittingham A, 1996) thus assisting the internalization of promastigotes and ultimately the host cell invasion. Although *Leishmania* are internalized, they can act as a 'Trojan horse' for impeding macrophage activation. Several strategies are utilized by promastigotes to exploit macrophages, such as interfering with intracellular signal transduction

pathways or delaying the phagosome-lysosome fusion as examples. via secreted molecules, incuding LPG (+PPGs). These mechanisms allow the promastigotes to be protected from lysosomal hydrolases until the environmental stimuli signaled transformation to highly resistant, obligatory intracellular amastigotes is undertaken (Desjardins M, 1997).

Astonishingly amastigotes, while residing in the newly formed, strongly acidified (pH 4.7-5.2), endosomal/lysosomal hydrolase-rich parasitophorous vacuoles, in contrast to most bacterial, fungal or protozoan pathogens, do not only survive, but indeed able to multiply (Alexander J, 1975; Antoine JC, 1990; Prina E, 1990; Lang T, 1994; Russell DG, 1995a,b), even if they do not express the protective macromolecule, LPG (Pimenta F, 1991). Host cells, besides serving as Trojan horse of parasites at hiding from the immune system, they also assist the spread of parasites when they migrate throughout the body. For successful parasitization, *Leishmania* need to intervene macrophage activation, a process that normally via production of high levels of nitric oxide and reactive oxygen intermediates leads to intracellular killing of invaders (Liew FY, 1990; Stenger S, 1994, 1996; Murray HW, 1999). *Leishmania* seem to interfere with macrophages on various ways such as inhibition of the iNOS induction and consequent NO production (Proudfoot L, 1995, 1996; Piedrafita D, 1999; Liew FY, 1990), inhibition of NADPH oxidase activation and consequent reactive oxygen production (reviewed in Descoteaux A, 1993) or scavenging free radicals (Chan J, 1989) that are assumed to be mediated by parasite glycoconjugates like the glycoinositolphospholipids (GIPLs) or LPG.

3.5.2 Hijacking the adaptive cellular defense mechanisms of the immune system by *Leishmania*

The mechanism how *Leishmania* can derail normal host adaptive immune responses has been investigated for decades. According to our current understanding, mostly based on infection models of inbred mice strains with *L. major*, the resistance or susceptibility to *Leishmania* infection, likewise to many intracellular pathogens, also largely depends on the type of CD4+ T-helper (T_H) cell response, while CD8+ cytotoxic T cell and B cell responses play somewhat limited role. Indeed these experiments, as model system, were the first *in vivo* demonstration of the relevance of T-helper 1/ T-helper 2 (T_H1/T_H2) balance in disease outcome (Sacks D, 2002 and references therein). As it was shown, if during the course of infection the differentiation of CD4+ T_H cells is polarized toward a dominating T_H1 cell response, then the lesion usually heals rapidly via induction of macrophage activation and consequent intracellular killing of *Leishmania*. On the other hand, if T_H2 response predominates, which favors a largely humoral immune response, the infection intends to persist, spread and lesions become chronic in the lack of macrophage activation (Fig.4).

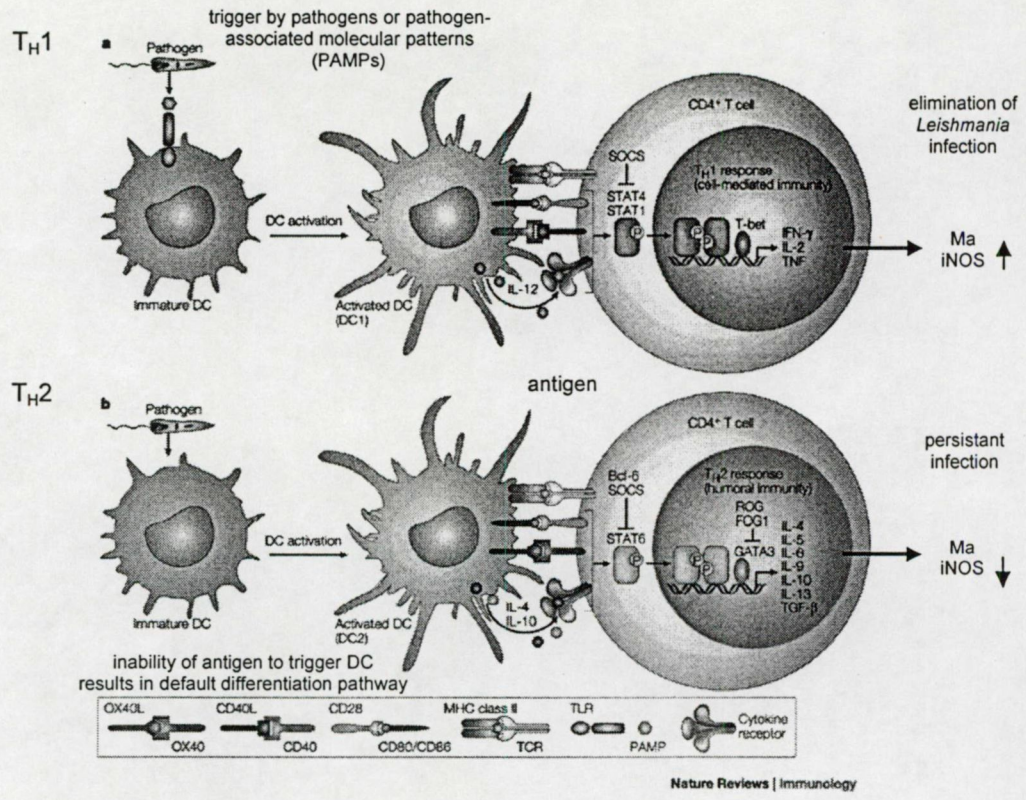


Fig.4. T_H1/T_H2 balance and the outcome of leishmaniasis (adapted from Sacks D, 2002).

The early events of infection in susceptible (e.g. Balb/c, a model of non-healing lesions of human leishmaniasis) or resistant (e.g. C57Bl/6, a model of healing human lesions) mice are rather similar and includes early production of interleukin-4 (IL-4) and other type-2 cytokines by CD4+ T cells (Stetson DB, 2002). However, later response becomes polarized towards T_H1 or T_H2 dominance that is highly dependent on the presence or lack of IL-12 produced by the infected macrophages (Heinzel FP, 1993; Sypek JP, 1993). Late events in susceptible mice include maintenance of T_H2 response in the lack of proper IL-12 stimulation that parallels with production of IL-4, IL-13, IL-10 and TGF-β, which prevents eventually the macrophage activation and parasite killing (Bogdan C, 2000). When a proper IL-12 stimulation is present, likewise observed in resistant mice, high levels of IFN-γ and TNFα are produced that upregulates iNOS expression and activates intracellular killing in the infected macrophages, that eventually leads to eradication of parasites and healing of the lesion (Bogdan C, 1996).

The role of *Leishmania* surface or secreted glycoconjugate molecules in shifting the immune response toward T_H2 dominance at non-healing lesions is being heavily investigated and likely stays in focus of interest for a while, since better understanding of these mechanisms can facilitate the development of preventive vaccines.

3.6 *Leishmania* genetics

Leishmania are unicellular diploid eukaryotes that reproduce asexually by division, therefore exhibit a rather clonal population structure (Tibayrenc M, 1991). However, the plasticity of their genome, including chromosomal rearrangements and DNA amplification is a well-known phenomenon (reviewed by Segovia M, 1997). This can ensure the essential heterogeneity for *Leishmania* population, which besides allowing flexible adaptation to changing natural environments also advantageous for coping with artificial evolutionary selection pressure, such as the drugs when via for example gene amplification *Leishmania* can develop drug resistance.

Besides the nuclear DNA, approximately 15% of the *Leishmania* genome is packed in a single mitochondrion, the kinetoplast. The kinetoplast DNA, which corresponds to the mammalian mitochondrial DNA, is replicated not separately, but together with the nuclear DNA in a coordinated manner.

Among other peculiar features of the Leishmania genome, some notably characteristic ones are the complete lack of introns and extensive sets of collinearly arranged genes, which result in gigantic polycistronic primary transcripts that are further trans-spliced and polyadenylated to monocistronic mRNAs whose steady state levels are individually regulated by post-transcriptional mechanisms (Myler PJ, 1999).

During the last decade, the molecular geneticist's arsenal for dissecting genetically *Leishmania*, increased reasonably with respect to a variety of positive and negative selectable markers, reporter genes, episomal and integrating expression vectors, highly efficient gene replacement by homologous recombination and transposable elements that have been developed and used extensively for reverse genetic approaches (summarized in Beverley SM, 1998; Clayton CE, 1999). Moreover, the Leishmania Genome Network initiative, that was established about a decade ago with the aim to sequence the genome of *L. major* (Friedlin strain), as model organism, for facilitating *Leishmnaia* research and consequently disease control strategies, has been accomplished in 2005 (Ivens AC, 2005).

4. Surface glycocalyx components and glycoconjugate virulence factors of *Leishmania*

Leishmania species synthesize vast amounts of glycosylated cell surface or secreted molecules, which form a highly resistant glycocalyx shield that serves as a multifunctional protective barrier during their life cycle. The molecular composition of the glycocalyx is highly

dependent on the stage of their life cycle. Promastigotes are shielded with a 20-40 nm thick layer of highly glycosylated molecules that predominantly consist of unusual glycoinositolphospholipids (GIPLs), a unique family of phosphoglycan-modified macromolecules that encompasses lipid-linked (lipophosphoglycan or LPG), protein-linked (proteophosphoglycans or PPGs) and unlinked forms (phosphoglycans or PGs), additionally highly conserved protein-linked glycosylphosphatidylinositol (GPI) membrane-anchors, and glycoproteins with uncommon N-linked glycans. The intracellular amastigotes, in contrast to promastigotes, lack such a thick glycocalyx layer and are abundantly coated with a layer of GIPLs and different lipid containing molecules acquired from the host cell. Investigating the role of these abundant mannose- and galactose-rich glycoconjugates in parasite survival and successful invasion of host and vector organisms has been in the focus of *Leishmania* research for the past decade.

4.1 Lipophosphoglycan (LPG)

In particular, the major cell surface glycoconjugate of *Leishmania*, LPG has been the most thoroughly studied molecule to date. Promastigotes express about 1–5 x 10^6 copies of LPG per cell, while amastigotes do only about 100 or 1000 copies that is moreover structurally distinct from the promastigote one (Glaser TA, 1991; Moody SF, 1993). This glycolipid macromolecule generally consist of four domains: a conserved membrane anchor of 1-O-alkyl-2-lyso-phosphatidyl(myo)inositol, followed by a conserved core structure of diphosphoheptasaccharide, and variable number (20-40) of phosphodisaccharide repeats with species- and stage-specific side chain modifications, and capped with a variable, dominantly mannose-rich structure (Fig.5; McConville MJ, 1993a). The membrane anchor domain of LPG predisposes the molecule to be membrane bound; however, LPG can be shed from the parasite surface and released to the extracellular space (Handman E, 1984; King DL, 1987), a mechanism that may allow *Leishmania* to get rid of glycocalyx bound host-derived defensive factors. LPG is believed to be a key molecule of *Leishmania* virulence in the mammalian host, with numerous functions attributed to it, such as conferring promastigote complement resistance in the mammalian host (Sacks DL, 1989), mediating attachment and uptake of promastigotes by macrophages (Handman E, 1985; Da Silva RP, 1989; Talamas-Rohana P, 1990), shielding against macrophage phagolysosomal hydrolases (Turco SJ, 1992) and inhibiting respiratory burst (Chan J, 1989) most likely via inhibition of host cell signal transduction pathways of protein kinase C (reviewed in Descoteaux A, 1993; Giorgione JR, 1996) and intracellular calcium (Eilam Y, 1985). Furthermore, the inhibition of phagolysosome maturation in macrophages (Desjardins M, 1997) and the transendothelial migration of monocytes (Ho JL,

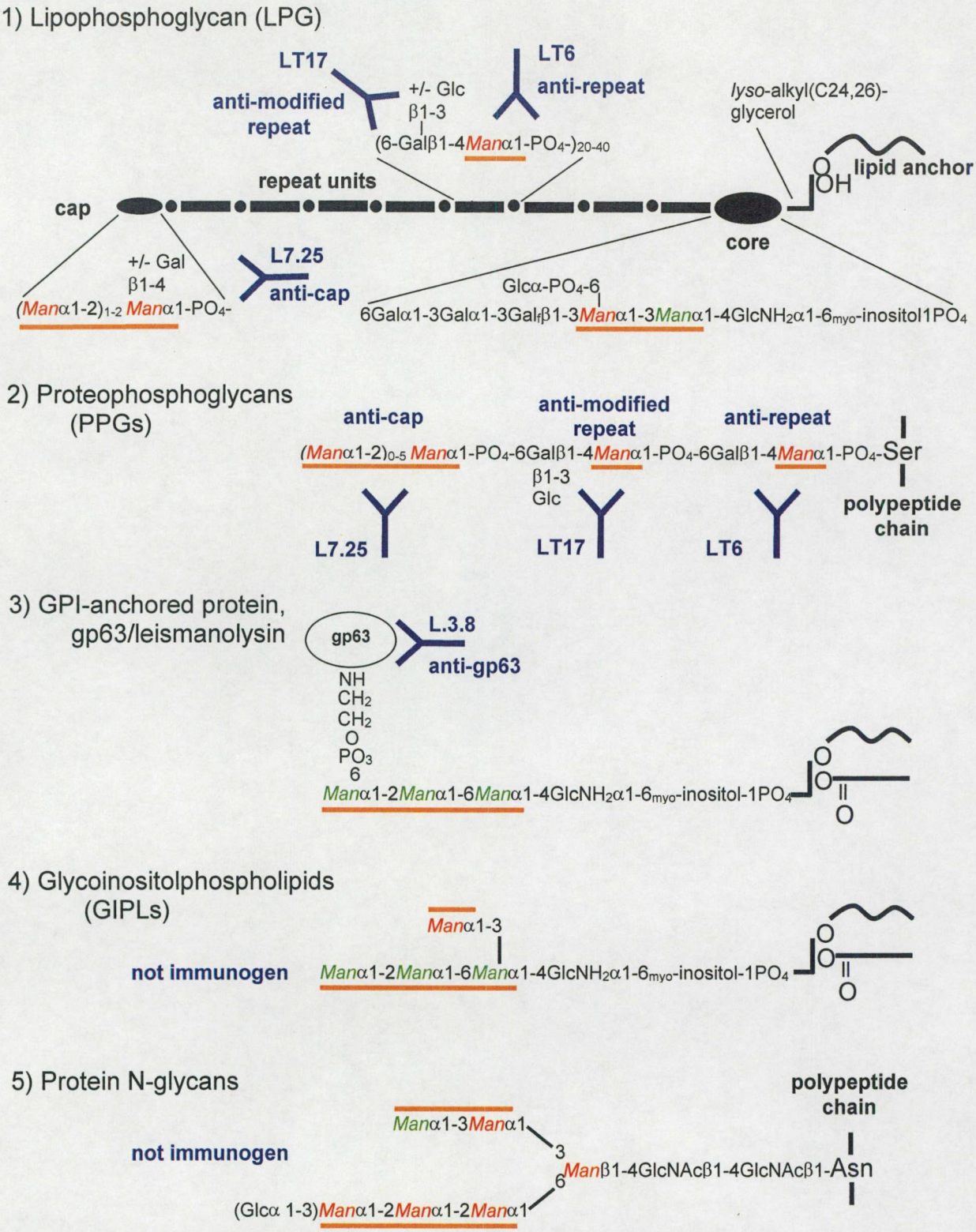


Fig.5. Mannose-containing glycoconjugates of *Leishmania* with epitops for monoclonal antibodies and concanavalin A binding sites applied in our studies, as well as the source of mannose residues (GDP-Man or Dol-P-Man activated mannose donor) indicated.

1996; Lo SK, 1998), suppression of interleukin (IL)-1β and tumour necrosis factor-α, likewise downregulation of inducible NO synthase (iNOS) and inhibition of macrophage IL-12 (Proudfoot L,

1996; Piedrafita D, 1999) considered to be key events of *Leishmania* infection mediated by LPG. The parallels drawn between these functional studies and data showing the loss of infectivity observed for LPG-deficient spontaneous mutant of *L. major* (Handman E, 1986) or chemical mutagenesis generated *L. donovani* or *L. major* (King DL, 1988; Elhay M, 1990; McNeely TB, 1990) reinforced the role of LPG as multifunctional virulence factor in *Leishmania*, although these strains may indeed carry rather uncharacterized mutations because of the nature of mutagenesis. Moreover, the final prove for such an overall role of LPG in *Leishmania* infectivity, via generating revertants that would reexpress LPG and consequently may have restored infectivity, was never shown (Beverley SM, 1998). Furthermore, some described 'LPG-deficient' *L. donovani* mutants (Descoteaux A, 1995, 1998) have defects more than LPG biosynthesis, since their proteophosphoglycan (Ilg T, 2000a; PPG reviewed in Ilg T, 2000b; see also in next section) synthesis is also affected.

Above observations, underline the necessity of applying state-of-art technologies of targeted gene replacement, instead of mutagenesis, for specifically generating and reverting *Leishmania* mutants in studies that aim to clarify the role of *Leishmania* glycoconjugate virulence factors.

Such a targeted gene replacement approach was utilized recently in *Leishmania* for the elucidation of the role of the glycoconjugate virulence factor LPG, when lpg1 and lpg2 genes that are involved in LPG synthesis were targeted. *Lpg1* gene encodes a putative β-galactofuranosyl [β-Gal_f] transferase localized in the Golgi apparatus and known to be crucial for the addition of an unusual internal β-Gal_f residue to the LPG diphosphoheptasaccharide core (Huang C, 1993; de Lederkremer RM, 1995). *Lpg2* gene encodes a Golgi GDP-mannose transporter (Ma D, 1997) involved in both, LPG and PPG biosynthesis.

Targeted gene replacement of *L.major lpg1* resulted attenuated parasites in line with the described role of LPG (Spath GF, 2000). However, in contrast to the former report, targeted deletion of *lpg1* gene in *L. mexicana* did not affect the virulence of parasites to macrophages or mice (Ilg T, 2000a). Several explanations may exist for this inconsistent finding: (i) for *L. major* β-Gal_f may be important for the biosynthesis of macromolecules other than LPG, which is indeed suggested (McConville MJ, 1993b), and therefore the *lpg1* mutant phenotype would not correspond to an exclusive lack of LPG, but also other glycoconjugates. (ii) LPG may have slightly different importance at the phase of transmission and/or establishment of *Leishmania* infection, which is suggested by the delayed onset of mouse pathology with *L. major lpg1* mutants versus *L. mexicana* ones that are fully virulent.

Observations with different Leishmania species, when *lpg2*gene was targeted are similar. *L. mexicana* knockouts lacking *lpg2* exhibited a 'glycotype' of deficient phosphoglycan repeat motifs,

which affects not only LPG, but also PPGs and free phosphoglycans. Although lacking 'major virulence factors', these *L. mexicana* mutants remained fully infectious to macrophages or mice (Ilg T, 2001). In *L. major*, the lack of *lpg2* gene led to strong attenuation of the parasites, although, in contrast to a former report of the same group which stated *lpg2* deficient *L. major* parasites being avirulent (Descoteaux A, 1995) these mutants were able to persist in the host (Spath, 2003), therefore potentially may develop pathology (especially in the case of HIV coinfection). This later risk was indeed shown recently by the reisolation of emerging, still *lpg2* negative, but highly infectious parasites from mice (Spath, 2004).

As demonstrated by the above studies, targeted gene replacement may facilitate to answer specific questions related to *Leishmania* virulence that can also potentially lead to elucidation of delicate differences between different *Leishmania* species.

4.2 Proteophosphoglycans (PPGs)

Since LPG was shown not to be the 'one and only' glycoconjugate that is essential for *Leishmania* virulence, recently the attention shifted to a novel class of highly glycosylated *Leishmania* glycocalyx components that emerge as key contributors to virulence, namely the proteophosphoglycans (PPGs). PPGs are a heterogeneous, rapidly expanding family of highly glycosylated *Leishmania* proteins that undergo extensive post-translational modification by phosphoglycan chains similar to those of LPG (Fig.5; reviewed in Ilg T, 2000b). Different *Leishmania* species show slightly diverse glycan structures on PPGs, similar to the species- and stage specificity described for LPG. Some PPGs in contrast, to LPG, are also expressed in the amastigote stage, which certainly entitles them being attractive candidate virulence factors of the intracellular stage in the invaded host cells. To date the novel class of PPGs has been most thoroughly studied in *L. mexicana*.

Promastogote PPGs include secreted acid phosphatases (SAPs), filamentous PPG (fPPG), membrane bound GPI-anchored PPG (mPPG) and promastigote PPG2 (pPPG2), while amastigotes express mPPG and secrete large amounts of amastigote PPG (aPPG).

SAPs exhibit a non-specific acid phosphatase activity and is released to the extracellular space via the flagellar pocket (Gottlieb M, 1982; Lovelace JK, 1986; Bates PA, 1989). *L.mexicana* secreted SAP is a complex comprising dominantly SAP1 (100 kDa) and minor amounts of SAP2 (200 kDa), which are encoded by lmsap1 and lmsap2 genes respectively (Ilg T, 1991a, b; Ilg T, 2003). The two proteins are almost identical except for some Ser/Thr-rich repeats, however their phosphoglycan structures are reasonably different (Ilg T, 1993, 1994; Wiese M, 1995). The phosphoserine-linked glycan chains of SAP1 are rather short (15-20nm) and consist mostly α1-2-

oligomannoside caps with or without a single repeat unit, while those of SAP2 are longer (ca. 100nm) phosphoglycans that are capped (Ilg T, 1994). The secreted SAP forms a millipede-like structure of remarkable length (2μm) via polymerization of subunits that can be well visualized by electron microscopy (Stierhof YD, 1998). In fact, the synthesis of polymerized SAP molecules is an enigma, since normally they are supposed to be assembled intracellularly in the Golgi apparatus. Instead of this rather unlikely scenario, the hypothesized place for the assembly of giant SAP macromolecule is the flagellar pocket, where they can reach an about 1000-fold higher concentration than in the medium that can well promote polymer formation (Stierhof YD, 1994). The function of SAPs is not well understood yet. The observation with *L. mexicana* that SAPs are not required for parasite growth or infectivity to macrophages and mice, implies that SAP function may be more important in the sandfly vector digestive tract, where via their low substrate specificity SAPs may have an important nutritional role (Ilg T, 1995, 1999a; Wiese M, 1995, 1998).

fPPG is a filamentous heavily phosphoglycosylated (ca. 96% w/w of total weight) 6μm long macromolecule. Over half of its protein backbone made of Ser residues, in addition to Ala and Pro, and almost 90% of those are phosphoglycosylated with around three phosphodisaccharide-long repeats that are variably transferase-modified and capped at the end (Ilg T, 1999a). This composition of fPPG leads to a filamentous structure with a diameter of 3-6 nm that confers proteinase resistance to the macromolecule (Ilg T, 1999b). fPPG is secreted from the flagellar pocket of *Leishmania* promastigotes and based on its physicochemical properties is able to form a gel-like network (Stierhof YD, 1994). This mucin-like three-dimensional mesh or gel plug can obstruct the digestive tract of infected sandfly vector (Stierhof YD, 1999) and consequently can enhance the transmission of parasites via the regurgitation and multiple blood feeding (a mechanism also known as 'blocked fly hypothesis', Rogers ME, 2004). Although the role of fPPG in the mammalian host is less understood, it was shown that fPPG inhibits the production of tumor necrosis factor-alpha and synergizes with interferon-gamma to stimulate the production of nitric oxide by macrophages (Piani A, 1999).

mPPG is a membrane bound PPG, attached via a GPI-anchor at the C-terminal domain (Ilg T, 1999a). Both promastigotes and amastigotes express mPPG, however the later ones do in much lower amounts (Piani A, 1999). The about 380 nm long protein chain encoded by the ppg1 gene includes several Leu-rich repeat (LRR) homologous regions in the N-terminal domain, that are implicated in protein-protein interaction as well as modulating signal transduction, and numerous (ca. 100) acceptor sites for phosphoglycosylation (Ilg T, 1999a; Piani A, 1999). mPPG function is not well described yet, but its unique structure, its predominant expression in promastigote stage, and its phosphoglycan structures shared with LPG may allow us to consider some hypotheses.

Likely mPPG can far extend out from the promastigote gylcocalyx that makes this macromolecule highly exposed to the environment. This feature would dedicate mPPG to interact with sandfly midgut, macrophages, and complement components, such functions that were formerly uniquely attributed to LPG. Although the copy number of mPPG is much lower than that of LPG, but mPPG LRR domain interaction with complement receptor 3 (CR3) (Kedzierski L, 2004) and the presence of about 800 Ser-linked phosphoglycan chains for receptor or complement-binding, versus the single binding site carried by LPG, makes highly feasible that mPPG has a significant contribution to *Leishmania* infectivity. mPPG may also play a role in parasite attachment to macrophages and in the modulation of macrophage function at the early stage of infection (Piani A, 1999).

Promastigotes secrete another PPG, the promastigote PPG2 (pPPG2) that is structurally distinct from SAP or fPPG/mPPG, but seems to share the same polypeptide backbone with a sereted amastigote PPG (aPPG) that both expressed from the ppg2 gene (Ilg T, 1995; Klein C, 1999). Although the polypeptide chain of 40-50nm length seems to be the same, its glycosylation appears to be stage-specific that can lead to remarkable differences in physicochemical properties and function between pPPG2 and aPPG (Klein C, 1999; Gopfert U, 1999). pPPG2 is glycosylated by either single capped monophosphorylated glycan or simple manno-oligosaccharides (Klein C, 1999), while aPPG displays amastigote-specific highly branched glycan structures of six mono- or multiphosphorylated glycans with four oligosaccharide caps (Ilg T, 1998). The function of pPPG2 is still unknown, although several potential roles are assumed for aPPG. These include the inhibition of antigen presentation by the complex glycan chains, and assisting this way at parasite hiding from the host immune system. Furthermore, similarly to other polyanionic compounds, aPPG can lead to lysosome-like vacuolization in macrophages, and contributing this way to the formation of parasitophorous vacuoles. Morover, aPPG can deplete the host complement system via activating strongly the mannose-binding lectin pathway and consequently saving released amastigotes from potential complement lysis.

It must be also noted that above functions of PPGs are partially inferred from the high similarity of their phosphoglycan motifs to other *Leishmania* glycoconjugates.

4.3 Phosphoglycans (PGs)

The supernatant of in vitro cultured *Leishmania* promastigotes contains a hydrophilic phosphoglycan made of capped oligosaccharide repeat units identical to those found on LPG, but without the GPI anchor and the glycan core (Turco SJ, 1992). Their structure exclude the possibility of PI-PLC–mediated release from LPG. PGs, although their function is still elusive, were shown to be secreted from the flagellar pocket to the extracellular space (Ilg T, 1999b).

4.4 GPI-anchored proteins

The glycocalyx of *Leishmania* promastigotes contains other abundant GPI-anchored proteins, than the formerly discussed mPPG, such as the major surface protease (MSP, also known as leishmanolysin or gp63), and the parasite surface antigen 2 (PSA-2)(Murray PJ, 1989).

Leishmanolysin/gp63 is a highly conserved multigene family encoded 63-kDa zinc metalloproteinase that is GPI-anchored to the cell surface via a myristic acid containing anchor (Fig.5). This major cell surface glycoprotein of *Leishmania* promastigotes covers the entire cell with a copy number of 500,000 per cell that accounts for about 1% of total cellular proteins. In amastigote stage, leishmanolysin/gp63 lacks the GPI-anchor, and it is present at much lower levels and most of it localized to the flagellar pocket (Medina-Acosta E, 1989). Leishmanolysin/gp63 contains three potential glycosylation sites (Button LL, 1988) that are N-glycosylated with high mannose-type biantennary glycan structures optionally with α1,3 linked terminal glucose (Olafson RW, 1990 ; Funk VA, 1997). The two most common N-glycan structures found in promastogotes are Man6GlcNAc2 and GlcMan6GlcNAc2, while amastigote N-glycans are more variable. The importance of stage specific glycosylation, likewise the role of gp63 in parasite life cycle is not completely understood, although some functions were identified for gp63. These include, the proteolytic degradation of host macromolecules, protection from complement lysis, facilitating parasite binding to macrophages via complement components (Alexander J, 1992). Simultaneous targeted gene replacement for all genes of gp63 multigene family was not performed, although a recent study generated mutants lacking gp63 in an elegant way by simply knocking out GPI8, a GPI-protein transaminidase that certainly led to the concomitant absence of other GPI-anchored proteins as well. Surprisingly these mutants grow normally, infectious to macrophages and mice, a finding that suggest that gp63 is not essential for *Leishmania* growth and infectivity (Hilley JD, 2000).

Parasite surface antigen 2 (PSA-2) is an either membrane bound or secreted macromolecule that contains leucine-rich repeat (LRR) motifs, likewise PPG does. The first functional role of PSA-2 has just been recently elucidated via hypothesizing parallel functions of PSA-2 and PPG based on their structural similarities (LRR motifs). In that study, *Leishmania* PSA-2 LRRs were shown also to bind to macrophages via CR3 (Kedzierski L, 2004). These findings prove that parasite attachment and invasion of macrophages involve a third *Leishmania* macromolecule, PSA-2, besides the better described PPGs and LPG.

4.5 Glycoinositolphospholipids (GIPLs)

Free GPIs or GIPLs, are major low molecular weight glycolipid constituents of *Leishmania* glycocalyx that were shown to be expressed at nearly constant levels throughout *Leishmania* life cycle, in contrast to other major parasite glycoconjugates, such as LPG, PPGs, and GPI-anchored proteins that are strongly downregulated in amastigote stage (McConville MJ, 1993a, 1994; Beverley SM, 1996; Ferguson MA, 1999 and references therein). GIPLs are expressed on both promastigote and amastigote surfaces at very high copy numbers of about 10^7 molecules/parasite and are neither protein nor polysaccharide-bound (Fig.5). Classification of GIPLs is based on their glycan headgroups those may be structurally related to protein GPI anchors (type I GIPLs) with Man 1,6Man 1,4GlcN 1,6-PI structure, or to LPG anchors (type II GIPLs) with Man 1,3Man 1,4GlcN 1,6-PI structure, or they can carry branched structures of the formerly mentioned ones based on a Man 1,6(Man 1,3)Man 1,4GlcN 1,6-PI motif (hybrid type GIPLs). The lipid components of the hybrid and type I GIPLs are rich in alkyl-acyl-PI with shorter (C18:0) alkyl chains, while type II GIPLs are more heterogeneous and contain longer alkyl chains (C24:0 or C26:0). The structure of different GIPLs is known in detail, however not that much is known about their function. Until recently, it was speculated that GIPLs are important virulence factors of the amastigote stage and believed to be involved in modulating signaling events in macrophages, such as NO synthesis and the oxidative burst. This notion was based on the observed abundant expression of GIPLs in amastogote stage, in contrast to other glycoconjugates. Additional observation that may support this hypothesis is that ectopic expression of *T. brucei* GPI-specific phospholipase C (GPI-PLC) in *Leishmania* leading to consequent depletion of GPI-anchored gp63, and several GIPLs, but not that of LPG, shows a phenotype exclusively in amastigotes that is characterized by reduced growth rate and infectivity to macrophages (Mensa-Wilmot K, 1994, 1999).

4.6 Lipid containing molecules acquired from the host

Besides self-synthesized lipid containing molecules, *Leishmania* can acquire different host molecules, ranging from cholesterol to various complex glycoshingolipids that can make up 20% or 50% of endogenous *Leishmania* pools, respectively. Resident host lipids in the lumen of parasitophorous vacuole may spontaneously intercalate into the amastigote plasma membrane or optionally after endocytosis and restructuring, being recycled to the plasma membrane. These host-derived molecules may give additional advantage to the amastigotes for hiding from host defense mechanisms (Ginger ML, 1999, 2000, 2001; McConville MJ, 1991; Winter G, 1994; Tripathi A, 2003; Schneider P, 1993; Puentes SM, 1988).

4.7 N-linked glycans

Leishmania has an uncommon N-glycan structure of GlcMan6GlcNAc2 (Fig.5), which has been most thoroughly studied in the GPI-anchored surface protease gp63. Our present knowledge about the role of N-glycans in *Leishmania* virulence or survival is rather limited. However, protein N-linked glycans are hypothesized to contribute to *Leishmania* virulence, based on the observation that tunicamycin-resistant *Leishmania* that has increased GlcNAc transferase activity due to gene amplification, loose their virulence more slowly and infect macrophages more efficiently than their wild type counterparts do (Kink JA, 1987).

5. Rationales and aims of the study

Cumulative evidence of large number of studies over the past 20 years suggests that mannose-containing glycoconjugates are required for *Leishmania* viability and virulence in every phase of their life cycle (Annex III. ref 1, 8, 10, and 17 and references therein). It is therefore surprising that the investigation of mannose activation pathway for glycoconjugate synthesis started only very recently.

The structure and biosynthesis of *Leishmania* glycoconjugates has been thoroughly studied (Annex I. ref 2-6) and their glycan structures has been elucidated, which predominantly consists of repeating phosphodisaccharides of mannose and galacotose, and smaller amounts of GlcNAc, $GlcNH_2$, Glc, D-Ara, and *myo*-inositol. These phosphoglycan structures seem to exhibit reasonable extent of structural and functional redundancy between different glycoconjugate classes. Therefore, one may hypothesize a special importance of given redundant glycan motifs instead that of single glycoconjugates of *Leishmania*. This notion is supported by experiences with LPG and gp63, and certainly raises questions about the rational of targeting simply particular virulence factors of *Leishmania* for dissecting its virulence (see section 4.1 Lipophosphoglycan (LPG)).

The building blocks for the assembly of different glycan motifs are the activated nucleotide sugar donors, such as GDP-Man, Dol-P-Man, UDP-Gal, UDP-Glc, and GDP-D-Ara (Annex I. ref 6-10). Given the high biosynthesis rate of the abundantly expressed glycoconjugates, the existence of a large and/or rapidly rechargeable pool of activated nucleotide sugar precursors can be assumed. Therefore, the mannose supply, which can be fueled from either external or internal sources, is predicted to have prime importance for *Leishmania* parasites.

In yeast and in mammalian cells the activation of mannose from Man-6-PO_4 to GDP-Man or Dol-P-Man donors, requires the sequential enzymatic action of phosphomannomutase (PMM), GDP-mannose pyrophosphorylase (GDPMP), and Dol-P-Man synthase (DPMS). (Fig.6) Man-6-PO_4 is a central metabolite in this pathway and may be supplied in two different ways. If exogenous Man

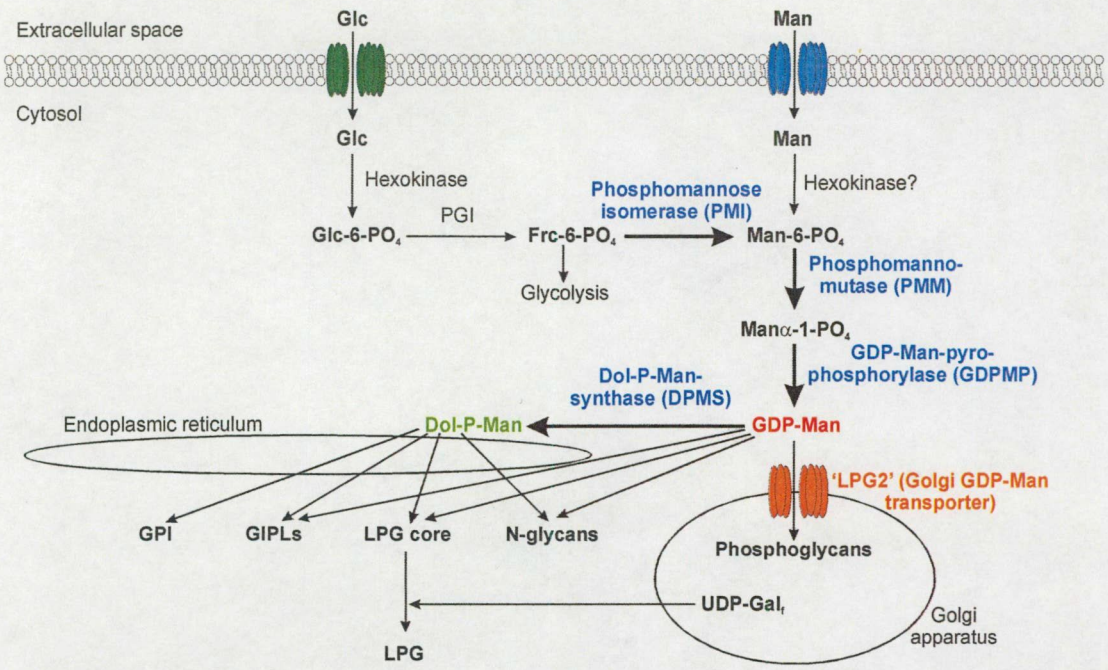


Fig.6. Schematic model of mannose activation pathway for *Leishmania* glycoconjugate biosynthesis.

is available, it can be taken up by glucose transporters (Annex I. ref 11) or the more specific mannose transporter, and immediately phosphorylated by hexokinase to form Man-6-PO_4 (Fig.6; Annex I. ref 12). Alternatively, in the hexose-monophosphate pool, Frc-6-PO_4 can be converted to Man-6-PO_4 in a reaction catalyzed by phosphomannose isomerase (PMI) (Fig.6; Annex I. ref 13). The lack of the latter enzyme leads to a conditional lethal phenotype in yeast (Annex I. ref 14) and a severe metabolic disease in humans (Annex I. ref 15-17), which demonstrates the essential role of PMI for these organisms. Moreover, genetic deficiencies of the downstream members of this enzyme cascade, such as PMM, GDPMP or DPMS, were described leading to devastating, systemic disorders of humans that is caused mostly by a single allele deficiency (heterozygosity) of the gene encoding these enzymes (Jaeken J, 2001). Complete deficiencies of these enzymes in humans considered incompatible with life.

Despite of its assumed central importance in glycoconjugate and glycan biosynthesis, the mannose activation pathway in *Leishmania* remained unexplored. However, its elucidation may have high scientific interest, especially in the context of identification of new potential drug targets for developing novel anti-parasitic medicines.

Therefore, we dissected by reverse genetic tools via targeted gene replacement the enzyme cascade of mannose activation pathway in *Leishmania mexicana* as a model organism. This species was chosen, since the structures of their different glycoconjugates are very well described that allowed us the most detailed assessment. In our studies, we aimed to assess the impact of mannose

activation pathway on *Leishmania* viability, growth, glycoconjugate composition of glycocalyx, and the virulence of parasite with the expectation to identify potential drug targets. We also aimed to establish a relationship between glycoconjugate composition and virulence in *Leishmania* that may facilitate the development of vaccines for the control of leishmaniasis.

6. Results and Discussion

6.1 Elucidating the role of phosphomannose isomerase (PMI) in *Leishmania mexicana*

Phosphomannose isomerase (PMI) catalyzes the reversible interconversion of fructose 6-phosphate (Frc-6-PO_4) and mannose 6-phosphate (Man-6-PO_4), which is the first step in the biosynthesis of activated mannose donors required for the biosynthesis of various glycoconjugates (Fig.6). To investigate the role of PMI for parasite glycoconjugate synthesis, in *Leishmania mexicana*, the gene encoding PMI (*lmexpmi*) was cloned, and gene deletion mutants (*Δlmexpmi*) generated. The phenotype of *Δlmexpmi* mutants was characterized with emphasis on their viability, growth, glycoconjugate expression and virulence to macrophages and mice.

6.1.1 Generation of *L. mexicana* mutants lacking PMI and characterization of their growth

The gene, *lmexpmi*, encoding *L. mexicana* PMI was cloned based on homology to phosphomannose isomerase genes of other eukaryotic organisms including *L. major*, and sequenced, as a prerequisite of targeted gene replacement (Annex I., Fig. 2). The *lmexpmi* gene turned out to be present as a single copy gene in the genome of *L. mexicana* and was expressed at very similar levels in both promastigotes and amastigote stages (Annex I., Fig. 4A and B). Double-targeted replacement of *lmexpmi* gene was performed in *L. mexicana* promastigotes by state-of-art homologous recombination technology utilizing PCR amplified *lmexpmi* gene replacement cassettes (Annex I., Fig. 3). Surprisingly *Δlmexpmi* clones could be generated without additional mannose supplementation, in contrast to formerly identified auxotrophic yeast mutants (Payton MA, 1991). The absence of *lmexpmi* in *L. mexicana Δlmexpmi* mutants was confirmed by Southern blot (Annex I., Fig. 3B). Consistently, in *L. mexicana Δlmexpmi* mutants PMI enzyme activity could not be detected, while wide type parasites exhibited high PMI activity that even exceeded more than 15-fold the mammalian PMI activities (Annex I., Fig. 4C, Annex I. ref 36), a level that is comparable to those found in the pathogenic fungi, C. albicans (75 milliunits/mg of protein, assay at 37 °C) (Annex I. ref 37).

For further experiments to characterize *Δlmexpmi* mutants, *lmexpmi* gene addback mutants were generated on *Δlmexpmi* mutant background with heterologous episomal expression vector (*Δlmexpmi*+pX*lmexpmi*) or alternatively with homologous recombination cassettes into the ribosomal locus (*Δlmexpmi*+pRIB*lmexpmi*) containing *lmexpmi* to show that the described features solely can be attributed to the lack of *lmexpmi* gene.

In vitro cultured *Δlmexpmi* mutants were still able to multiply in standard growth medium, even when traces of Man were removed by extensive dialysis, although their growth was slower in comparison to wild type or *lmexpmi* addback (*Δlmexpmi*+pX*lmexpmi*) controls, a phenotype that could be reversed with exogenous mannose supplementation (0.2 mM)(Annex I., Fig. 5). It is surprising that in contrast to *S. cerevisiae*, *Aspergillus nidulans*, and *C. albicans*, wherein loss of PMI activity is lethal unless Man is provided, *Δlmexpmi* are viable and can multiply without exogenous mannose supply (Annex I. ref 14, 38, 39). Indeed this *Δlmexpmi* phenotype seems to be unique, because complete absence of PMI has never been observed in vertebrate cell lines. Moreover, only partial lack of PMI was observed in a human hereditary disease, termed congenital disorder of glycosylation (CDG) type Ib that further strengthens the incompatibility of complete PMI absence with life (Annex I. ref 40).

Additional experimental observations showed that further increase in mannose concentrations could progressively slow down the growth of *Δlmexpmi* mutants, while it did not affect control strains. This stress ultimately at a mannose concentration of 10mM led to killing of most *Δlmexpmi* parasites (Annex I., Fig. 5) that resembles to a phenomenon based on toxic accumulation of Man-6-PO_4 and possible ATP depletion, known as honeybee syndrome (Annex I. ref 41).

6.1.2 *L. mexicana Δlmexpmi* mutants exhibit impaired glycoconjugate synthesis that can be reversed by exogenous mannose supplementation or *lmexpmi* gene addback

Although PMI is not indispensable for *Leishmania* viability, its lack leads to profound changes in (phospho-)glycan content of *Leishmania* glycoconjugates that was shown in our studies by immunoblot, flow cytometry and immunofluorescence microscopy.

Δlmexpmi promastigotes grown in standard medium were unable to synthesize phosphoglycan repeat-modified LPG and PPGs (Annex I., Fig. 6A lane 2-5, 6B, 7A, B, 8A-H), while *L. mexicana Δlmexlpg1* that are specifically defective in LPG synthesis (Annex I. ref 19), expressed still mPPG (Annex I., Fig. 6A lane 6). Despite the fact that LPG absence supposed to

increase the accessibility to the GPI-anchored leishmanolysin/gp63 on the surface of *Leishmania*, described in *Δlmexlpg1* and *Δlmexlpg2* mutants (Annex I. ref 19, 32), the expression of this glycoprotein was decreased in *Δlmexpmi* mutants (Annex I., Fig. 7D). Furthermore, in *Δlmexpmi* mutants a general underglycosylation of glycoproteins were suggested by the decrease in surface binding sites for concanavalin A (Annex I., Fig. 7C, 8R), and by electrophoretic mobility shifts of many cellular proteins including secreted SAP (Annex I., Fig. 6C, D). Finally, the abundant promastigote GIPLs iM2, iM3, iM4, and EPiM3 (Annex I. ref 28) were down-regulated to undetectable levels (Annex I., Fig. 9), while metabolic [^{3}H]$GlcNH_2$ labeling revealed new, more hydrophobic GIPL species in *Δlmexpmi* mutants that were not seen in wild type controls. These novel GIPLs believed to represent precursors of GIPL biosynthesis, such as $GlcNH_2$-phosphatidylinositol or GlcNAc-phosphatidylinositol species. In contrast, to a recent observation that describes mannose-containing GIPLs as essential molecules for *L. mexicana* viability (Annex I. ref 42), *Δlmexpmi* mutants remained viable, while their mannose-containing GIPLs were downregulated to undetectable levels.

The above-described phenotype of *Δlmexpmi* mutants can be restored by *lmexpmi* gene reexpression or exogenous mannose supplementation (200 μM) to the growth medium for bypassing the existing metabolic blockage, a fact reinforces that the observed phenotype can be attributed to the lack of *lmexpmi* gene.

6.1.3 *L. mexicana Δlmexpmi* mutants are attenuated although remain infectious to macrophages and mice

At last, the potential of *Δlmexpmi* mutants for infecting macrophages *in vitro* and developing lesions in mice *in vivo* was tested in a sensitive (Balb/c) mice strain.

L. mexicana Δlmexpmi was less efficient in establishing and maintaining an infection in mouse peritoneal macrophages than the parental wild type strain (Annex I., Fig. 12A). The synthesis of phosphoglycan repeat epitopes by macrophage residing intracellular *Δlmexpmi* parasites was downregulated, but surprisingly still detectable, compared to wild type controls (Annex I., Fig. 13, compare E-H with A-D).

In sensitive Balb/c mouse infection model, inoculation of *L. mexicana Δlmexpmi* into the footpad led to much smaller lesions compared to wild type parasites, but remarkably these mutants remained infectious (Annex I., Fig. 12B, C).

Episomal or, in particular, chromosomally integrated addback of the *lmexpmi* gene to *Δlmexpmi* mutants improved their ability to persist and multiply within macrophages and led to increased virulence to BALB/c mice (Annex I., Fig. 12).

The fact that *Δlmexpmi* mutants, although severely attenuated, are still infectious to macrophages and mice is at first unexpected, because the general impairment of glycoconjugate synthesis (Annex I. ref 2), in particular the defect of GIPL assembly (Annex I. ref 43), supposed to preclude virulence completely. The remarkable observation although that *in vitro* cultured *Δlmexpmi* do not express phosphoglycan repeats (Annex I., Fig. 8B, F), unless exogenous mannose is supplemented, while macrophage internalized intracellularly residing *Δlmexpmi* do express them (Annex I., Fig. 13E-H), suggests that amastigotes must have access to host-derived mannose of unknown origin within the parasitophorous vacuole that may also be sufficient for remaining infectious.

Taken together, this study shows that deletion of *lmexpmi* in *L. mexicana* leads to dramatic downregulation of glycoconjugate synthesis (the phosphoglycan repeats [-6-Galβ1-4Manα1-PO_4-], the surface expression of the abundant GPI-anchored leishmanolysin, and GIPLs) and that surprisingly these mutants are viable, in contrast to a recent report (Annex I. ref 22). Moreover, *Δlmexpmi* mutants, although attenuated, remain infectious to macrophages and mice, a remarkable phenotype that likely can be attributed to a bypass of the glycosylation defect by host-derived mannose.

The observation that *lmexpmi* is not indispensable for viability, implies that downstream disruption of mannose activation pathway to PMI may also be compatible with life.

6.2 Elucidating the role of phosphomannomutase (PMM) in *Leishmania mexicana*

Mammalian and fungal PMM catalyzes the reversible interconversion of Manα1-PO_4 and Man-6-PO_4 (Fig.6). PMM requires Manα1,6-bis-PO_4 as cofactor (Annex III. ref 32) and belongs to a novel family of phosphotransferases with its conserved DXDX(T/V) motif. The first aspartic acid in this sequence is involved in phosphate transfer and is transiently phosphorylated (Annex III. ref 4). *S. cerevisiae* PMM (Sec53p) was initially identified in a screen for mutations with defects in the secretory pathway (Annex III. ref 23). In humans, two enzymes (PMM1 and PMM2) with PMM activity have been cloned and characterized (Annex III. ref 27, 28). While PMM1 exhibits also a potent phosphoglucomutase activity, PMM2 is specific for Man-PO_4 and appears to be the dominant PMM in most human tissues (Annex III. ref 36).

To investigate the role of PMM in *Leishmania*, the *Leishmania mexicana* gene encoding PMM (*lmexpmm*) was cloned, and gene deletion mutants (*Δlmexpmm*) were attempted to be generated to characterize them phenotypically with emphasis on viability, growth, glycoconjugate expression and virulence to macrophages and mice.

6.2.1 Generation and growth characteristics of *L. mexicana* mutants lacking PMM

The *lmexpmm* gene, encoding *L. mexicana* PMM (*LmxPMM*), was homology cloned based on the sequence of other eukaryotic phosphomannomutases, and subsequently sequenced to perform targeted gene replacement of *lmexpmm*. Southern blot analysis of *L. mexicana* genomic DNA suggested that *LmxPMM* is a single copy gene (Annex III., Fig. 4A). RT-PCR revealed that *LmxPMM* mRNA is present in both parasite life stages but it is more abundant in mammalian host residing amastigotes (Annex III., Fig. 4B). However, the enzyme itself seems to be equally abundant in both life stages (Annex III., Fig. 5A) and largely (>95%) soluble in the cytosol (Annex III., Fig. 5B), a finding that was also supported by immunofluorescence experiments (data not shown).

Deletion of PMM gene in *S. cerevisiae* is lethal (Annex III. ref 23). Partial PMM2 deficiency in humans leads to the severe disease of congenital disorders of glycosylation (CDG) type Ia that is characterized by underglycosylation of many proteins and severe encephalopathy leading to psychomotor retardation (Annex III. ref 34). Remarkably, among hundreds of mutations, none has been identified that leads to complete abrogation of PMM2 activity, therefore complete lack of PMM is considered incompatible with human life.

Surprisingly targeted gene replacement of *lmexpmm* was successfully performed in *L. mexicana*, and a series of PMM mutant clones (*Δlmexpmm*) was isolated (Annex III., Fig. 4A). The absence of *LmxPMM* was confirmed by immunoblotting total-cell lysates (Annex III., Fig. 4C). These clones were viable in standard culture medium and showed only a mild growth defect compared to wild type parasites that defect as expected could not be rescued by exogenous mannose supplementation (Annex III., Fig. 6C).

In enzyme assays, *Δlmexpmm* mutants showed markedly lowered PMM enzyme activity, with less than 10% of wide type levels that likely derives from minor PMM activities of other mutases like phosphoglucomutase or phospho-N-acetylglucosaminemutase (Annex III., Fig. 6A).

6.2.2 Overall downregulation of mannose-containing glycoproteins and glycolipids in *L. mexicana Δlmexpmm* mutants that can be reversed by *lmexpmm*

gene addback

The product of *LmxPMM*, Manα1-PO_4, is the substrate for GDP-Man formation that directly or indirectly is the sole mannose donor for glycoconjugate synthesis in *Leishmania* (Annex III. ref 22 and references therein; Fig.6). Therefore, the lack of *LmxPMM* predicted to have broad effect on the synthesis of *Leishmania* glycoconjugates.

Δlmexpmm mutants indeed downregulated completely or strongly LPG, the phosphoglycan caps and repeats of PPGs as shown by different methods with the anti-phosphodisaccharide repeat mAb LT6, anti-phosphotrisaccharide repeat mAb LT17, and anti-phosphoglycan cap mAb L7.25 (Annex III., Fig. 4D-F, 7B, J,L). The lack of LPG was also confirmed by metabolic [^{3}H]inositol labeling, which experiment also shown absence of [^{3}H]inositol-labeled GPI-anchored gp63/leishmanolysin (Annex III., Fig. 5C). Downregulation of gp63/leishmanolysin was also confirmed by surface labeling (Annex III., Fig. 7D, E, K), suggesting that protein GPI anchor synthesis was also severely affected in *Δlmexpmm* mutant parasites. The abundant GIPLs were undetectable in *Δlmexpmm* by HPTLC of total lipids with or without metabolic labelings, while some novel GIPLs seemed to appear (Annex III., Fig. 5E-H). The presence of N-glycosylation defect in *Δlmexpmm* mutants was evidenced by mobility shift of the normally heavily N-glycosylated MBAP (Annex III., Fig. 5D; Annex III. ref 31, 41). Fluorescence microscopy and FACS showed extremely weak signal in *Δlmexpmm* with ConA (Fig. 7G, H, M), a lectin that strongly binds to α-Man residues (Annex III. ref 7) present in *L. mexicana* N-glycans, LPG, PPGs, and GIPLs (Annex III. ref 8, 16, 17, 21), a finding that suggests an overall downregulation of mannose-containung glycoconjugates. Hexose analysis by sensitive gas chromatography-mass spectrometry confirmed this observation, since the amount of macromolecule-associated mannose (all known lipid- and protein-bound mannose-containing glycoconjugates) in *Δlmexpmm* mutants was below the detection limit (less than 1% of wild-type levels)(Annex III., Fig. 6D).

The low-level expression of GPI-anchored gp63, mannooligosaccharide caps, and ConA binding sites in *Δlmexpmm* mutants assayed by sensitive immunochemical techniques and lectin binding, may be explained by the leakiness of mutants attributed to the detected minimal PMM activity of other enzymes that may lead to synthesis of very limited amounts of Manα1-PO_4.

The glycosylation defects of *Δlmexpmm* can be reversed by *lmexpmm* gene addback, but in contrast to the PMI mutants, this phenotype cannot be rescued by exogenous mannose (Annex III., Fig. 4A, C-F, 5C, D, H, 6C, 7C, F, I, J-M).

6.2.3 Loss of virulence phenotype of *L. mexicana Δlmexpmm* mutants to

macrophages or mice

Δlmexpmm mutant promastigotes were unable to establish an infection in cultured macrophages (Annex III., Fig. 11A), which cannot be attributed to lack of attachment to or that of host cell invasion, because they were as efficiently taken up by macrophages as wild type counterparts, however *Δlmexpmm* mutants were eliminated soon after (not shown).

Furthermore, *Δlmexpmm* promastigotes proved to be avirulent to sensitive Balb/c mice, even at high parasite load (10^7/mouse) (Annex III., Fig. 11B, C). Attempts to reisolate *Δlmexpmm* parasites from inoculated animals were repeatedly unsuccessful, suggesting that the parasites cannot establish an asymptomatically persisting infection *in vivo*. Wild type level of virulence for *Δlmexpmm* mutants could be restored by *lmexpmm* gene addback either by chromosomal integration into the rRNA locus or by episome (Annex III., Fig. 11A, B, and C).

The loss of virulence may be explained by the inability of *Δlmexpmm* parasites to utilize exogenous mannose from host derived source for glycoconjugate synthesis, in contrast to *Δlmexpmi* parasites that retained this capacity and consequently their virulence (Annex III. ref 11).

The unique observation that *lmexpmm* is not indispensable for viability, implies that downstream disruption of mannose activation pathway to PMM could also be compatible with life. Therefore, we decided to dissect further the mannose activation pathway in *L. mexicana*, namely to study the GDP-mannose pyrophosphorylase (GDPMP) enzyme.

6.3 Studying the role of GDP-mannose pyrophosphorylase (GDPMP) in *Leishmania mexicana*

The consecutive action of phosphomannomutase (PMM) and GDP-mannose pyrophosphorylase (GDPMP) transforms the exogenous or hexomonophosphate pool-derived Man-6-PO_4 to GDP-Man, which is a key nucleotide sugar of mannose activation pathway that feeds the eukaryotic glycoconjugate synthesis (Fig.6; reviewed in Annex II. ref Freeze H, 1999). GDP-Man is the directly or indirectly utilized mannose donor for all mannosylation reactions in eukaryotes, moreover a precursor of GDP-fucose synthesis (Annex II. ref Kaufman RL, 1968) and substrate of ascorbic acid synthesis in plants (Annex II. ref Wheeler GL, 1998). The essential role of GDP-Man is supported by the observations describing a lethal phenotype of *Saccharomyces cerevisiae* and *Candida albicans*, when their genes encoding GDPMP, or other members of mannose activation pathway, are deleted (Annex II. ref Hashimoto H, 1997; Warit S, 2000; Kepes F, 1988; Orlean P, 1988). To date, no uni- or multicellular eukaryotic organisms were reported with complete absence

of GDPMP activity that suggests that complete disruption of the mannose activation pathway and the resulting absence of mannose-containing glycoconjugates are incompatible with eukaryotic life.

On the other hand, our observations in *Leishmania*, when almost abolished PMM enzyme activity (*Δlmexpmm*) was still compatible with life, although insufficient for maintaining the biosynthesis of mannose-containing glycoconjugates, suggested that in contrast to other eukaryotes and a recent report on the essential need of Dol-P-mannose in *Leishmania* (Annex III. ref 22), the lack of GDPMP may be compatible with life.

To test our hypothesis about of GDPMP in *Leishmania*, the gene encoding GDPMP (*lmexgdpmp*) was cloned, and gene deletion mutants (*Δlmexgdpmp*) were intended to be generated, with the hope to find a unique eukaryotic phenotype that can be characterized with special emphasis on viability, growth, glycoconjugate expression and in these parasites also for virulence.

6.3.1 Attempt to generate *L. mexicana* mutants lacking GDPMP

The *lmexgdpmp* gene, encoding *L. mexicana* GDPMP (*LmxGDPMP*), was homology cloned based on the sequences of other eukaryotic GDPMPs (Annex II., Fig. 1B).

Northern blotting and RT–PCR analysis suggest that *LmxGDPMP* mRNA is present in both parasite life stages, and somewhat more abundant in the intracellular amastigote forms (Annex II., Fig. 2D, E), although immunoblots of total cell lysates probed with affinity-purified antibodies against recombinant *LmxGDPMP* indicate equal abundance of *LmxGDPMP* in both life stages (Annex II., Fig. 3D). *LmxGDPMP* activity is largely (>90%) soluble (data not shown), which is in agreement with immunoblotting studies on membrane fractions (Annex II., Fig. 3E). Immunofluorescence experiments on permeabilized promastigotes using anti-*LmxGDPMP* serum gave a diffuse signal throughout the cell body, which is also in alignment with a cytoplasmic localization of *LmxGDPMP* (Annex II., Fig. 5C).

The single-copy *lmexgdpmp* was attempted to be knocked-out by targeted gene replacement, which was described to give rise lethal phenotype in *Saccharomyces cerevisiae* and *Candida albicans*.

Surprisingly, two rounds of targeted *lmexgdpmp* gene replacement using the antibiotic resistance markers HYG (hygromycin) and BLE (bleomycin) in *L. mexicana*, a eukaryotic organism, (Annex II., Fig. 2A) gave rise clones lacking both alleles of *lmexgdpmp* (*Δlmexgdpmp*)(Annex II., Fig. 2C) and the protein *LmxGDPMP* as well (Annex II., Fig. 3A). Therefore, remarkably in *Leishmania* the complete absence of GDPMP encoding gene, in contrast to other eukaryotes, results in viable mutants that showed only a mild mannose independent growth defect (Annex II., Fig. 4H).

Although *LmxGDPMP* is largely cytoplasm located, GDPMP enzyme assays gave strong GDP-Man-independent background reactions, probably due to different metabolites and/or enzymes present in crude extracts. Therefore, to compare *LmxGDPMP* activity in wild type and *Δlmexgdpmp* mutants, concentrated ultracentrifugation supernatants were fractionated by gel filtration to remove interfering components. While in wild type fractions, a prominent *LmxGDPMP* activity peaked between 240 and 300 kDa, no *LmxGDPMP* activity could be detected in *Δlmexgdpmp* mutants (Annex II., Fig. 4A). Determination of UDP-glucose pyrophosphorylase (UDPGP) enzyme activity in both chromatography runs served as control for equivalent loadings (Annex II., Figure 4A).

6.3.2 Overall downregulation of mannose-containing glycoproteins and glycolipids in *L. mexicana Δlmexgdpmp* mutants

Earlier studies suggested that in *L. mexicana*, like in other eukaryotes, GDP-Man is the sole mannose donor for glycoconjugate synthesis (Fig.6; Annex II. ref Mengeling BJ, 1997; Ilgoutz SC, 1999; Moss JM, 1999 and references therein). Therefore, in agreement with the foreseen impact of mannose activation pathway blockage for glycoconjugate synthesis, *Δlmexgdpmp* mutants do not express any of the known lipid- and protein-bound mannose-containing glycoconjugates. The *Δlmexgdpmp* phenotype was described applying different techniques including immunoblotting, immunofluorescence microscopy, FACS and metabolic labeling with radioactively labeled metabolites, and showed the following characteristics:

(I) absence of LPG and phosphodisaccharide glycan motifs on PPGs (Annex II., Fig. 3B, C, 5A I vs. II, V vs. VI, 5B LT6 and LT17) and also complete lack of anti-phosphoglycan caps shown by mAb L7.25 (Annex II., Fig. 4E), moreover the normally highly phosphoglycosylated secreted acid phosphatase (SAP) showed a strong mobility shift detected by affinity purified anti-SAP antibody, which cannot be reversed by exogenous mannose (Annex II., Fig. 4D, F and G).

(II) absence of the dominant GPI-anchored surface protein gp63 (Annex II., Fig. 5A, IX, X), although it could be detected within permeabilized cells, particularly in the perinuclear region, which suggested its retention in the endoplasmic reticulum for quality control reasons (Figure 5A, XI). Furthermore, the cross-reactive determinant (CRD), which is indicative of GPI-anchored proteins in many eukaryotes, including *L. mexicana* (Annex II. ref Zamze SE, 1988; Ilg T, 1993), was not present in GDPMP promastigote total cell lysates after GPI-phospholipase C (GPI-PLC) digestion (Annex II., Fig. 3G, lanes 3 and 4), and no [^{3}H]inositol-labeled gp63 was detected after metabolic labeling (Annex II., Fig. 4C), suggesting that protein GPI anchor synthesis is severely downregulated in the mutant parasites.

(III) defect in N-glycosylation shown by the strong mobility shift (15–20 kDa) of the normally heavily N-glycosylated, but neither GPI-anchored nor phosphoglycosylated, membrane-bound acid phosphatase (MBAP) (Annex II. ref Menz B, 1991; Wiese M, 1996) (Annex II., Fig. 3F, lanes 1 and 2).

(IV) undetectable level of mannose-containing GIPLs examined either by orcinol staining or fluorography after metabolic labeling with [^{3}H]Man, [^{3}H]GlcNH2 and [^{3}H]myo-inositol in high-performance thin-layer chromatography (HPTLC)-separated total lipids (Annex II., Fig. 6).

(V) general lack of α-Man residues shown by the absent signal for the strong α-Man binding lectin, concanavalin A (ConA) by fluorescence microscopy or FACS analysis that suppose to detect *L.mexicana* N-glycans, LPG, PPGs and GIPLs (Annex II. ref Ilgoutz SC, 1999; Ilg T, 2000a,b; Ilg T, 2001) (Annex II., Fig. 5A, XIII, XIV, and B ConA).

(VI) finally, hexose analysis of trifluoroacetic acid (TFA)-hydrolyzed promastigote membranes by high-pH anion-exchange high-pressure liquid chromatography (HPLC) showed that Man is absent (<1%) in macromolecules (Annex II., Fig. 4B), a result also confirmed by hexose analysis using gas chromatography–mass spectrometry (<0.5% of wild-type levels; A.Garami, T.Ilg, A.Mehlert and M.A.J.Ferguson, unpublished results).

The fact that the above described *Δlmexgdpmp* mutant phenotype with profound glycosylation defects could be reversed by *lmexgdpmp* gene addback (Annex II., Fig. 2B and C) – leading to reconstitution of all glycoconjugates investigated in this study (Annex II., Fig. 3A-C, F, lanes 3; 3G, lanes 5, 6; 5A, IV, VIII, XII, XVI, B), although the re-expressed enzyme level was only ~10–20% of the wild-type levels (Annex II., Fig. 3A and data not shown) – suggests that the *Δlmexgdpmp* mutant phenotype was caused by the absence of *lmexgdpmp* gene.

6.3.3 Loss of virulence phenotype of *L. mexicana Δlmexgdpmp* mutants to macrophages or mice

Δlmexgdpmp mutant promastigotes were totally unable to establish an infection in cultured macrophages (Annex II., Fig. 7A). This was not due to the lack of attachment or that of host cells invasion, as demonstrated by time course experiments, wherein the *Δlmexgdpmp* mutants after internalization were rapidly killed, whereas wild-type parasites survived (Annex II., Fig. 7B) and proliferated (data not shown).

Furthermore, *L. mexicana Δlmexgdpmp* promastigotes proved to be completely avirulent to Balb/c mice, even at a high parasite load (1×10^7/mouse) (Annex II., Fig. 7C, D). Moreover, the repeated attempt to re-isolate GDPMP parasites from the site of injection, the draining lymph nodes

or the spleen of challenged animals was unsuccessful that suggests the absence of persisting parasites.

Virulence of *Δlmexgdpmp* mutants to both, macrophages and mice, could be completely restored by *lmexgdpmp* gene reexpression (episomal or rRNA locus integrated) (Annex II., Fig. 7A, C and D).

Uniquely, this work is the first demonstration of a viable eukaryotic organism with complete disruption of mannose activation pathway and the concomitant loss of all known mannose-containing glycoconjugates. *Δlmexgdpmp* mutants downregulate LPG, PPGs, mannose-containing GIPLs, GPI-anchored proteins and mannose-containing N-glycans to levels undetectable by different sensitive methods. In *Δlmexgdpmp* mutants, *LmxGDPMP* activity is undetectable, and no evidence supports the existence of an alternative pathway for GDP-Man synthesis. If such a hypothetical pathway would exists in *L.mexicana*, it must be extremely inefficient.

Taken together, the exclusive observation that *lmexgdpmp* is not indispensable for *Leishmania* viability, when no traces of *LmxGDPMP* enzyme activity could be detected, in contrast to the slightly 'leaky' *Δlmexpmm* mutants, strongly suggests that downstream disruption of mannose activation pathway should also be compatible with life. Therefore, in contrast to a recent report in *L. mexicana* (Annex III. ref 22), the deletion of dolicholphosphate-mannose synthase gene (*lmexdpms*) would be expected to give rise viable parasites with largely unaffected biosynthesis of PPGs and N-glycan structures (Fig.5).

To test our hypothesis the role of dolicholphosphate-mannose synthase (DPMS) was studied in *L. mexicana.*

6.4 The role of dolicholphosphate-mannose synthase (DPMS) in *Leishmania mexicana*

DPMS is an endoplasmic reticulum associated enzyme that catalyzes the formation of dolicholphosphate-mannose (Dol-P-Man), the second major activated mannose donor for glycosylation reactions (Fig.6). Two different types of DPMS exist in eukaryotes: the enzymes of *S. cerevisiae*, *Trypanosoma brucei*, and *Leishmania mexicana* are formed by a single polypeptide chain, while DPMS from mammals, worms, and *Schizosaccharomyces pombe* is composed of three subunits (Annex III. ref 26). Mutation in human DPMS leads to congenital disorders of glycosylation (CDGs) type Ie, which is characterized by underglycosylation of many proteins and severe encephalopathy leading to psychomotor retardation (Annex III. ref 34). Remarkably, in CDG type Ie cases, a residual DPMS activity is always detectable, therefore the complete lack of DPMS is

considered to be incompatible with human life (Annex III. ref 37), just as a report suggested it for *L. mexicana* (Annex III. ref 22).

6.4.1 Attempt to generate *L. mexicana* mutants lacking DPMS

DPMS is an essential enzyme for *S. cerevisiae* (Annex III. ref 33) and *S. pombe* (Annex III. ref 5). The gene encoding *L. mexicana* DPMS (*lmexdpms*) has recently been cloned and sequenced, and it has been reported that this enzyme is essential for *L. mexicana* (Annex III. ref 22). However, the successful generation of *L. mexicana* PMI, PMM, and GDPMP gene deletion mutants led us to the conclusion that in contrast to this previous report, *LmxDPMS* may also not be required for *L. mexicana* viability.

In agreement with this notion, after cloning the locus of *L. mexicana* single-copy DPMS gene by PCR amplification, two rounds of targeted gene replacement (Annex III., Fig. 3A) resulted in parasite clones lacking both alleles of *LmxDPMS* ORF (*Δlmexdpms*) (Annex III., Fig. 8A). In *Δlmexdpms* promastigotes, the 29-kDa *LmxDPMS* protein band was no longer detectable (Annex III., Fig. 8C). To assess DPMS activity of *Δlmexdpms* mutants, [^{14}C]Man transfer from GDP-[^{14}C]Man into the lipid fraction of resuspended microsomal pellets was measured that showed a lack of signal for *Δlmexdpms* mutants in comparison to wild type controls (Annex III., Fig. 6B). Immunoblottings on subcellular fractions showed that *LmxDPMS* is likely a membrane-associated protein (Annex III., Fig. 9B), as it was also predicted by its DNA sequence (PMMref22). *LmxDPMS* mRNA appeared to be expressed at higher levels in amastigotes, while its protein product showed higher levels in promastigote stage (Annex III., Fig. 8B, 9A).

6.4.2 Downregulation of selected mannose-containing glycoproteins and glycolipids in *L. mexicana Δlmexdpms* mutants

In vitro studies on microsome fractions suggested that Dol-P-Man is the α-mannose donor for the following glycoconjugates in *L. mexicana*: the first mannose residue of LPG core sequence, all three mannose residues of protein GPI anchors, the first two mannose of GIPLs, and possibly the sixth mannose (Man6) of N-glycans. By contrast, the biosynthesis of phosphoglycan chains on both LPG and PPGs and the synthesis of Man1-5 of N-glycans require exclusively GDP-Man, so does not depend on Dol-P-Man (Fig.6 and 7; also Annex III., Fig. 1A, B; Annex III. ref 22).

In agreement with the above model, the *Δlmexdpms* mutants showed the following phenotype:

(I) lack LPG, as indicated by immunoblots (Annex III., Fig. 8D, E), metabolic labeling with [^{3}H]myo-inositol (Annex III., Fig. 9C), immunofluorescence microscopy with MAb LT6 (Annex III., Fig. 10A, B, B'- the secretory organelle located weak signal appearing at longer exposure is most likely due to the secretion of phosphoglycosylated SAP and fPPG (Annex III. ref 39), whose glycosylation is not affected by the lack of *LmxDPMS*), and by unsuccessful attempts of purifying LPG by standard methods (not shown) (Annex III. ref 29).

(II) the phosphoglycan repeat and mannooligosaccharide cap synthesis of proteins, in contrast to LPG, remains normal or is even slightly elevated, as shown by immunoblottings of total-cell lysates (Annex III., Fig. 8D-F) or culture supernatant containing the major secreted PPGs, SAP and fPPG (data not shown).

(III) the biosynthesis of GPI anchors defective in the mutants, shown by undetectable surface expression of the GPI-anchored metalloproteinase gp63/leishmanolysin (Annex III., Fig. 10D, E, 10K), and the lack of [^{3}H]myo-inositol label incorporation into gp63 or any other *Leishmania* proteins (Annex III., Fig. 9C, lanes 1 and 2). The intracellular staining that appears in permeabilized *Δlmexdpms* mutants (Annex III., Fig. 10E') suggests an endoplasmic reticulum localization of gp63 and may reflect the blockage of its intracellular trafficking because of insufficient GPI-anchor linkage.

(IV) N-glycosylation does not seem to be greatly affected, since no significant electrophoretic mobility shift of the heavily N-glycosylated, although not phosphoglycosylated or GPI-anchored (Annex III. ref 31) membrane-bound acid phosphatase (MBAP), was observed (Annex III., Fig. 9D, lanes 1 and 2). Moreover, ConA surface binding was comparable in *Δlmexdpms* and wild-type promastigotes, as indicated by immunofluorescence microscopy (Annex III., Fig. 10G, H) and FACS analysis (Annex III., Fig. 10M).

(V) dominant Man-containing GIPLs iM2, iM3, and iM4 of wild-type parasites (Annex III. ref 30) are absent in *Δlmexdpms* promastigotes by orcinol staining of HPTLC-separated total lipids (Annex III., Fig. 9E, lane 1, 2) that was confirmed by [^{3}H]Man, [^{3}H]GlcNH2 (Annex III., Fig. 9F and G), and [^{3}H]myo-inositol (not shown) labelings.

(VI) hexose analysis by gas chromatography-mass spectrometry confirmed that the total mannose content of *Δlmexdpms* promastigote membranes is rather low, was only around 3% in comparison to wild type parasites (Annex III., Fig. 6D).

Reexpression of *lmexdpms* gene either by chromosomal integration into the ribosomal locus (Annex III., Fig. 3B, 8A, C-F lanes 3, 9C-G lanes 3, 10C, F, I, J-M) or introduction of episomal gene copies (Annex III., Fig. 10L and data not shown) led to reversion of the described glycosylation defects of LPG, protein GPI anchors, and GIPLs in *Δlmexdpms*.

6.4.3 Attenuated virulence phenotype of *L. mexicana Δlmexdpms* mutants to macrophages or mice

Although *L. mexicana Δlmexdpms* mutants lack LPG, GPI-anchored gp63 and mannose-containing GIPLs, unexpectedly still succeeded in colonizing host cells and showed only moderate decrease in infectivity to macrophages in comparison to wild type controls (Annex III., Fig. 11D). In Balb/c mice, the onset and progression of disease caused by *Δlmexdpms* was only marginally slower in comparison to the wild-type control strain, but eventually the animals failed to control the infection (Annex III., Fig. 11E). *Δlmexdpms* parasites could be reisolated from lesion tissue, draining lymph nodes and the spleen of inoculated animals, which confirms the *in vivo* persistence of *Δlmexdpms* mutants. The decrease in infectivity to macrophages and virulence to mice could be completely rescued by reexpression of *lmexdpms* gene (Annex III., Fig. 11D and E).

LmxDPMS has been reported to be essential for *Leishmania* (Annex III. ref 22). In contrast to this view, the existence of *L. mexicana* mutants that lack upstream key members (*LmxPMM* and *LmxGDPMP*) of mannose activation pathway suggested that, *LmxDPMS* should not be required for *L. mexicana* viability either. The successful generation of knockout parasites that lack *lmexdpms* when lacking DPMS activity, has proven this assumption. Unexpectedly, these mutants although lacking glycoconjugates that were described earlier on as major virulence factors (Annex III. ref 3, 8, 38), such as, LPG, Man-containing GIPLs, and the GPI-anchored surface metalloproteinase leishmanolysin (gp63), still remain infectious to macrophages and mice.

Taken together, the above observations indeed call for revisiting the role of *Leishmania* glycoconjugate virulence factors.

7. Conclusion

Solid body of evidence based on large number of studies over the past 20 years suggests that mannose-containing glycoconjugates are required for Leishmania viability and virulence throughout their life cycle (Annex III. ref 1, 8, 10, and 17 and references therein). The glycan structures of these *Leishmania* glycoconjugates predominantly consists of mannose and galacotose, and smaller amounts of GlcNAc, $GlcNH_2$, Glc, D-Ara, and *myo*-inositol. The building blocks for glycoconjugate biosynthesis are various activated nucleotide sugar donors, such as GDP-Man, Dol-P-Man, UDP-Gal, UDP-Glc, and GDP-D-Ara (Annex I. ref 6-10). Given the high rate of glycoconjugate synthesis in *Leishmania*, the proper mannose supply is predicted to be prime importance for these parasites. Therefore, our studies tried to elucidate the importance of mannose activation pathway by dissecting

it via reverse genetics, and estimate the role that of for *Leishmania* viability, growth, glycoconjugate virulence factor synthesis, and virulence that eventually may lead to potential novel treatments of leishmaniasis.

The mannose activation pathway that is well described in yeast and mammalian cells and generates GDP-Man and Dol-P-Man donors for glycosylation, requires the sequential enzymatic action of phosphomannose isomerase (PMI), phosphomannomutase (PMM), GDP-mannose pyrophosphorylase (GDPMP), and Dol-P-Man synthase (DPMS). Partial deficiency of any enzymes on this cascade were described as devastating genetic disorders of humans, moreover complete loss of enzyme function is considered being incompatible with life.

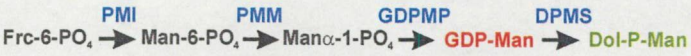
In our studies in *Leishmania mexicana* – as one of the best-studied model species of *Leishmania* for glycoconjugate composition, synthesis, and structure – the genes of mannose activation pathway were homology cloned and sequenced with the aim to generate stepwise mutants lacking members of this enzyme cascade at different levels.

Remarkably, in contrast to different pathogenic fungi, yeast species or humans, – wherein functional lack of mannose activation pathway, leading to lack of glycoconjugates, is incompatible with life – in *Leishmania*, targeted gene deletion mutants were generated successfully without major difficulties and they turned out to be viable without traces of mannose supplementation to the medium. These results doubtlessly show that mannose-containing glycoconjugates, in contrast to a former report in *L. mexicana* (Annex II. ref Ilgoutz SC, 1999), are not essential for *Leishmania* viability, although the *in vitro* growth of mutants was slightly affected. In the case of *Δlmexpmi* mutants, this growth phenotype can also be reversed to normal by exogenous mannose supplementation, which confirms that *Leishmania*, likewise other eukaryotes, also can have mannose from two sources, an internal one from the hexose-monophosphate pool or external uptake via mannose transporter, in line with our current understanding of the mannose activation pathway (Fig.6).

As it was anticipated, the mutants lacking components of mannose activation pathway show complex phenotype in terms of surface glycoconjugates and virulence (Table 1).

The deletion of *lmexpmi* leads to dramatic downregulation of glycoconjugate synthesis, namely LPG, the phosphoglycan repeats [-6-Galβ1-4Manα1-PO_4-] of PPGs, the surface expression of GPI-anchored gp63/leishmanolysin, and mannose-containing GIPLs, that can be reversed to normal by exogenous mannose supplementation.

The phenotype of *lmexpmm* knockout mutants was more remarkable, wherein the absence of *lmexpmm* leads to striking lack of protein and lipid glycosylation to such an extent that the mannose content of the membranes is undetectable (less than 1% of wild-type levels). Furthermore, mannose-



L. mex. strain	wild type	ΔPMI	ΔPMM	ΔGDPMP	ΔDPMS	ΔLPG1	ΔLPG2	ΔGPI18
LPG	norm	0	0	0	0	0	0	norm
Glyc. of PPGs	norm	0 or ↓↓↓	0	0	norm	norm	0	norm
GPI-anchored proteins (gp63)	norm	↓↓	0 or ↓↓↓	0	0	norm	norm	0
Mannose cont. GIPLs	norm	0	0	0	0	norm	norm	norm
N-glycans	norm	↓↓	0 or ↓↓↓	0	norm or (↓)	norm	norm	norm
virulence	+++	+	-	-	++	+++	+++	+++
reference	several	Garami and Ilg, 2001 JBC	Garami et al., 2001 Mol Cell Biol	Garami and Ilg, 2001 EMBO J	Garami et al., 2001 Mol Cell Biol	Ilg, 2000 EMBO J	Ilg et al., 2000 JBC	Hilley et al., 2000 Mol Biol Cell

Table.1. Glycosylation defects and virulence phenotype of *L. mexicana* mutants.

containing GIPL expression is also undetectable by radiolabeling techniques. Only sensitive immunochemical techniques and lectin binding assays detect low level expression of GPI-anchored gp63, mannooligosaccharide caps, and ConA binding sites in *Δlmexpmm* mutants. This slight leakiness of *Δlmexpmm* parasites may be explained by the synthesis of very limited amounts of Manα1-PO_4 via some residual PMM activity that may be attributed to other mutases, such as phosphoglucomutase or phospho-N-acetylglucosaminemutase.

The lack of next enzyme in the sequence, *LmxGDPMP*, leads to an even more remarkable phenotype characterized by the downregulation of all mannose-containing glycoproteins and glycolipids, such as LPG, PPGs, GPI-anchored proteins (gp63), mannose-containing GIPLs and N-glycans to undetectable levels. This work notably is the first demonstration of a viable eukaryotic organism with the complete disruption of mannose activation pathway and consequent loss of all known mannose-containing glycoconjugates. It must also be mentioned that at present, there is no

evidence for the existence of any alternative pathway for GDP-Man synthesis, and even if such a hypothetical pathway might exist in *L. mexicana*, it must be extremely inefficient.

Δlmexdpms mutants, lacking *LmxDPMS*, exhibit a complex phenotype described by selective lack of LPG, mannose-containing GIPLs, and the GPI-anchored leishmanolysin (gp63) that all have been described as major virulence factors in *Leishmania* (Annex III. ref 3, 8, 38), while N-glycosylation appears to be largely unaffected.

The virulence of all mannose activation pathway mutants to macrophages *in vitro* or to mice *in vivo* was affected, however to different extent. The *Δlmexpmi* and *Δlmexdpms* mutants remain infectious, although are attenuated, while *Δlmexpmm* and *Δlmexgdpmp* mutants appear to be avirulent.

The fact that the *Δlmexpmi* mutants, although severely attenuated, are still infectious, most probably can be attributed to a bypass of the glycosylation defect supplied by host-derived mannose. Furthermore, in contrast to *Δlmexpmi* mutants, the inability of *Δlmexpmm* and *Δlmexgdpmp* parasites to utilize exogenous mannose could be an explanation of their avirulent phenotype.

Based on recent data and the studies summarized in this thesis that apply *L. mexicana* as a model system, a comprehensive picture about the role and significance of mannose-containing glycoconjugates in *Leishmania* virulence is emerging (Table 1): we have shown earlier that LPG alone is not required for full virulence of *L. mexicana* (Annex III. ref 16). The absence of GPI-anchored proteins, including gp63 (Annex III. ref 15), and the lack of LPG and PPGs (Annex III. ref 21) is not abrogating virulence to mammals either. In *Δlmexpmi* mutants, although *in vitro* several mannose-containing glycoconjugates (LPG, phosphoglycan repeats of PPGs, GPI-anchored gp63 and mannose-containing GIPLs) are strongly downregulated, *in vivo* they remain infectious, but attenuated, because of a bypass effect by host-derived mannose that enables the invading parasites to reconstitute their surface molecules almost equivalent to that of wild-type levels. Even combined absence of LPG, GPI-anchored gp63, and mannose-containing GIPLs, as it was shown in *L. mexicana Δlmexdpms* mutants, does not lead to loss of virulence, only attenuation of these parasites. However, severe downregulation or absence of all mannose-containing glycoconjugates in *Δlmexpmm* or *Δlmexgdpmp* mutants, respectively, renders *Leishmania* unable to infect macrophages and mice.

The open question that remains after all: What are than the essential glycan components required for virulence? In contrast to the avirulent *Δlmexpmm* or *Δlmexgdpmp* mutants, the virulent *Δlmexdpms* parasites have normal capacity to synthesize PPG phosphoglycan repeats and caps, and their N-glycosylation appears to be largely unaffected. This fact nominates these molecules as major

candidates responsible for *Leishmania* virulence. On the other hand, the virulent *lmexlpg2* mutants lack phosphoglycans on PPGs, except for a small numbers of mannooligosaccharide cap epitopes (Table 1) (Annex III. ref 21). This leaves the latter structures and N-glycans on the agenda as potential determinants of *Leishmania* virulence.

Alternatively, it is conceivable that between the five different classes of *Leishmania* glycoconjugates (LPG, PPGs, protein GPIs, GIPLs, and N-glycans) a shared functional redundancy may exist in their contribution to virulence. Certainly, this would allow the parasites to lack one, two, or even more classes of glycoconjugate virulence factors without major impact on virulence, and only the absence of all classes could lead to complete loss of parasite virulence.

Since targeting single virulence factors did not seem to lead to breakthrough in unfolding the mystery of *Leishmania* virulence, based on our novel approach, when the pathway of glycoconjugate biosynthesis was targeted, a new paradigm is arising that strongly suggests that instead of a single pinpointed mannose-containing glycoconjugate, the various glycan motifs that share considerable structural and functional redundancy between different classes of glycoconjugates, may be responsible for evolutionarily successful virulence of *Leishmania*.

Our studies identify the mannose activation pathway of *Leishmania* as a virulence pathway that can be an attractive target for the development of novel anti-leishmanial drugs. Moreover, such a drug would be expected being effective more broadly against pathogens, since for example in pathogenic fungi the functional lack of the mannose activation pathway was shown leading to a lethal phenotype. The herein generated avirulent mutants most likely with suitable adjuvant may also be attractive subjects of vaccine development to prevent leishmaniasis (Garami A. unpublished results; Stewart J, 2005).

The remarkable observation that *Leishmania* is the first eukaryotic organism with complete blockage of mannose activation pathway and without any consequent effect on viability makes *L. mexicana* parasites a unique eukaryotic model system for studying mannose-containing glycoconjugate biosynthesis by reverse genetics. Certainly, this may also allow identifying various transferases that are responsible for the assembly of the redundant glycan motifs implicated in *Leishmania* virulence.

Taken together, these observations indeed envision a paradigm change of *Leishmania* virulence and call for revisiting the role of *Leishmania* glycoconjugate virulence factors based on the importance of shared glycan motifs. This road may lead us to a new horizon of understanding leishmaniasis, the disease and consequently to develop better disease control strategies, such as new medications and vaccine based prevention.

8. Materials and Methods (see in corresponding Annex)

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11. Annex of the Ph. D. thesis related publications of the author

Annex I. about *LmxPMI*

Attila Garami and Thomas Ilg: The role of phosphomannose isomerase in *Leishmania mexicana* glycoconjugate synthesis and virulence.
J. Biol. Chem. 2001 Mar 2; 276(9): 6566-75.

Annex II. about *LmxGDPMP*

Attila Garami and Thomas Ilg: Disruption of mannose activation in *Leishmania mexicana*: GDP-mannose pyrophosphorylase is required for virulence, but not for viability.
EMBO J. 2001 Jul 16; 20(14): 3657-66.

Annex III. about *LmxPMM* and *LmxDPMS*

Attila Garami, Angela Mehlert and Thomas Ilg: Glycosylation defects and virulence phenotypes of *Leishmania mexicana* phosphomannomutase and dolicholphosphate-mannose synthase gene deletion mutants.
Mol. Cell. Biol. 2001 Dec; 21(23): 8168-83.